IMMOBILISATION OF ENZYMES TO PERLOZA™ CELLULOSE RESIN

This thesis was presented in partial fulfilment of the requirements for the degree of Master of Science in Biochemistry at Massey University

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ABSTRACT

The studies reported in this thesis describe the use of Perloza™ beaded cellulose resin as a solid support for enzyme immobilisation via covalent binding. The aim of the project was to extend the uses for Perloza™ and to compare the use of well known solid support activation chemistries with a recently developed one for Perloza™. Preparations such as these have potential industrial uses. Three attachment chemistries were studied. The first activation employed 1,1-carbodiimidazole (CDI) then direct attachment of enzyme. The second again used CDI activation followed by attachment of a 6-aminocaproic acid spacer arm and then the enzyme. The final method used was attachment of a diol and subsequent oxidation to an aldehyde. The diol/aldehyde method had the advantage over the CDI methods of being based on aqueous chemistries. The two CDI based methods require extensive use of dry organic solvents. The enzymes investigated in this study were trypsin, chymotrypsin, α-amylase, horseradish peroxidase (HRPO) and alcohol dehydrogenase (ADH).

Trypsin was immobilised successfully by all three chemistries. All preparations retained significant activity after immobilisation at room temperature as judged by the chromogenic substrate specific for trypsin N-α-benzoyl-DL-arginine-*p*-nitroanilide.HCl (BAPNA). Measurable activity was retained in different studies from between 2 to 7 days at 60°C. The activity of immobilised trypsin with a synthetic peptide substrate was comparable to the activity of free trypsin with the same substrate.

Chymotrypsin was also successfully immobilised using all three chemistries. Each preparation showed significant retention of activity after immobilisation as judged by the chromogentic substrate N-glutaryl-L-phenylalanine-*p*-nitroanilide (GAPNA). Stabilisation to heating at 60°C was less successful than with trypsin but significant activity was still retained for between 3 and 6 hours. The activity of immobilised preparations with a peptide substrate was comparable to free chymotrypsin.

α-Amylase, horseradish peroxidase and alcohol dehydrogenase were studied less extensively than trypsin and chymotrypsin. Nevertheless all three enzymes were

successfully immobilised onto Perloza™-CDI-ACA and Perloza™-Diol/Aldehyde. Difficulty was encountered in achieving significant levels of any enzyme immobilisation to Perloza™-CDI for all three enzymes. Subsequent activity assays showed HRPO and α-amylase retained significant activity on all three resin preparations. ADH showed no measurable activity on Perloza™-CDI and very little activity on Perloza™-CDI-ACA and Perloza™-Diol/Aldehyde.

Investigations have shown that enzymes can be immobilised on Perloza™ with retention of significant amounts of normal activity at room temperature and improved stability compared with free enzyme at high temperature. Comparisons of the CDI activations with the diol/aldeyde chemistry showed better performance by the latter in trypsin immobilisation and similar performance for chymotrypsin immobilisation. Horseradish peroxidase and α-amylase were successfully immobilised using CDI/ACA and diol/aldehyde chemistries with the CDI/ACA giving higher initial specific activities than the diol/aldehyde preparation. Alcohol dehydrogenase was also successfully immobilised but gave no measurable activity.

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LIST OF ABBREVIATIONS

AAA	amino acid analysis
ACA	6-aminocaproic acid
ADH	alcohol dehydrogenase
BAPNA	benzoyl-DL-arginine-*p*-nitroanilide.HCl
BCA	bicinchoninic acid
BPNPG-7	p-Nitrophenyl maltoheptaoside
CDI	1,1'-carbonyldiimidazole
EDC	1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide hydrochloride
GAPNA	N-glutaryl-L-phenylalanine-*p*-nitroanilide
HCl	hydrochloric acid
HMP	4-hydroxymethylphenoxymethyl-copolystyrene-1% divinylbenzene resin
HPLC	high performance liquid chromatography
HRPO	horseradish peroxidase
$NaCNBH_3$	sodium cyanoborohydride
NAD^+	nicotinamide adenine dinucleotide (oxidised form)
NHS	N-hydroxysuccinimide
TFA	trifluoroacetic acid

CHAPTER ONE

INTRODUCTION

Enzymes

Enzymes (literally "in yeast") are polypepeptides and act as catalysts in biological processes (Dixon and Webb 1979). The unique catalytic properties of enzymes have been utilised in many different areas including food technology (Reed 1966), analytical chemistry, preparative organic chemistry, medicine, and preparative biochemistry. Advantages of the use of enzymes as catalysts include a high catalytic activity, a high degree of substrate specificity, activity at ambient temperature, normal pressure and in aqueous conditions (Klibanov 1983). Disadvantages in the use of enzymes include enzyme sensitivity to conditions such as extremes of heat and pH, proteolysis, and denaturing agents. They may be expensive due to scarcity, isolation and purification procedures. Difficulties can occur in the separation of an enzyme from a product leading to contamination, especially unacceptable in the pharmaceutical and food industries. Immobilisation of enzymes can in some cases solve some or all these problems (Melrose, 1971).

Enzyme Immobilisation

Immobilised enzymes are defined as "enzymes physically confined or localised in a certain defined region of space with retention of their catalytic activities, which can be used repeatedly and continuously" (Gerhartz, 1990). Immobilisation can allow easy separation of enzyme from products (Rosevear *et al,* 1987), give increased storage stability and in some cases alter favourably enzyme properties such as thermostablity and stability to denaturing agents and pH extremes. Processes using immobilised enzymes can be relatively easily controlled (Hayashi and Ikada, 1990; Rosevear *et al,* 1987). Possible disadvantages of immobilisation include problems with mass transfer (where there is diffusional resistance of product or substrate to or from the active site), reduced activity due to conformational changes, partitioning effects where

concentrations may differ from the bulk solution and microenvironmntal effects caused by the support used (Goldstein, 1976). In some cases enzyme structure and function can be altered due to immobilisation (Clark and Bailey, 1984).

Immobilisation Methods

The immobilisation of enzymes was first described in 1916 by Nelson and Griffin. They immobilised yeast invertase by adsorbtion to charcoal and showed it catalysed the hydrolysis of sucrose. A number of different methods have been used to immobilise a variety of enzymes. A small selection of the large number of examples available in the literature is shown in Table 1.1. The method of immobilisation selected depends on the enzyme involved and the end use. A variety of methods are available for immobilising enzymes (Table 1.2). These have been extensively reviewed in the literature (Rosevear *et al,* 1987; Mosbach, 1976 and 1987; Cabral and Kennedy, 1991). Enzymes can be attached by chemical or physical binding to an insoluble supporting matrix (carrier); included in this group are immobilisation by adsorption, ionic bonding and covalent binding. Enzymes may also be entrapped where they are free in solution but restricted to a limited space (Gerhartz, 1990). These methods may be used individually or in combination with each other

.

Physical Adsorption

One of the simplest methods for immobilisation is physical adsorption. "Adsorption is the adhesion of an enzyme to the surface of a carrier that has not been specifically functionalized for covalent attachment" (Messing, 1976). For immobilisation to occur the enzyme needs only to be brought into contact with the matrix, no reactive species are required. The enzyme is weakly attached to the support by interactions such as hydrogen bonding, hydrophobic interactions, ionic bonding or a combination of any or all of these. Little if any activity is lost, as enzymes are not deformed by strong bonding found in some other immobilisation methods. The weak bonding allows easy regeneration of the support. Disadvantages of protein binding by adsorption processes include facile desorption of enzyme in conditions of variable pH, high substrate concentration, and high ionic strength, due to the weak bonding of the enzyme to the

support. Ionic bonding gives a stronger linkage than hydrogen bonding and hydrophobic interactions, but weaker than covalent binding.

Immobilisation Method	Enzyme	Reference
Physical Adsorption	Trypsin	Mukherjea *et al* (1980)
Metal Link/Chelation	Amyloglucosidase	Cabral *et al* (1981)
Cross-linking	Chymotrypsin	Halling and Dunnill (1979)
Entrapment	Glucose Oxidase	Arica and Hasirci (1993)
Covalent Binding	Chymotrypsin	Sun *et al* (1996)

Table 1.1 Examples of Immobilised Enzymes

Metal-link/Chelation

Transition metals such as titanium and zirconium can form bonds with groups such as the hydroxyls found on polysaccharide matrices to give a complex ion. It can then form bonds with an enzyme to give an ionic link with amino acid side chains replacing the matrix group as ligands. The bonding is stronger than adsorption and similar to ionic bonding but weaker than covalent bonding. The preparation is relatively simple compared with covalent binding and the solid support can be regenerated (Kennedy and Cabral, 1995).

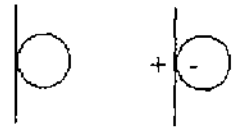
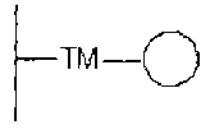
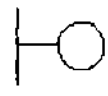
Immobilisation Method	Description
Physical Adsorption	 Enzyme bound to the support by physical interactions such as H-bonding or ionic bonding.
Metal Link/ Chelation	 Transition metals (T.M) linked to hydroxyl groups on the matrix and then linked to the enzyme
Cross-linking	 Enzymes form covalent bonds with each other via bi- and multifunctional reagents to form insoluble macromolecules.
Entrapment	 Enzymes trapped in a structure allowing substrate in and product out without releasing enzyme.
Covalent Binding	 Enzymes are attached via covalent bonds to solid supports.

Table 1.2 Immobilisation Methods

Cross-linking or Aggregation

Enzymes form covalent bonds with one another by reacting with bi and multi-functional reagents to form large insoluble macromolecules (Gerhartz, 1990). The multiple attachments found in this method usually deform the enzyme and block off many active sites causing a significant loss in activity.

Entrapment

In this method the enzyme is trapped within a structure such as a gel or membrane which allows substrate in and product out. It is not bound to the matrix as for adsorption, ionic, and covalent bonding. There are several types of entrapment depending on the type of structure containing the enzyme (Mosbach, 1976). These structures include gel matrices, microcapsules, liposomes, hollow fibres and ultrafiltration membranes. There is no activity loss due to enzyme modification from strong bonding such as is found in aggregation and covalent bonding. Proteases and high molecular weight inhibitors are prevented from entering but so are high molecular weight substrates. Supports are not renewable.

Covalent Binding

Covalent binding is the immobilisation method utilised in this study. Either the support is activated and then enzyme added or enzyme activated and then added to the support (Figure 1.1). The enzyme is linked via a covalent bond to a solid support to form a stable conjugate. The bonding is much stronger than for physical adsorption and ionic bonding, so enzyme leakage is minimised. Loss of activity due to enzyme modification and blocking of the active site can also be minimised, as the multiple attachments found in cross-linking are not required. Some activity, however, is usually lost either by modification or blocking of the active site (Mosbach, 1976).

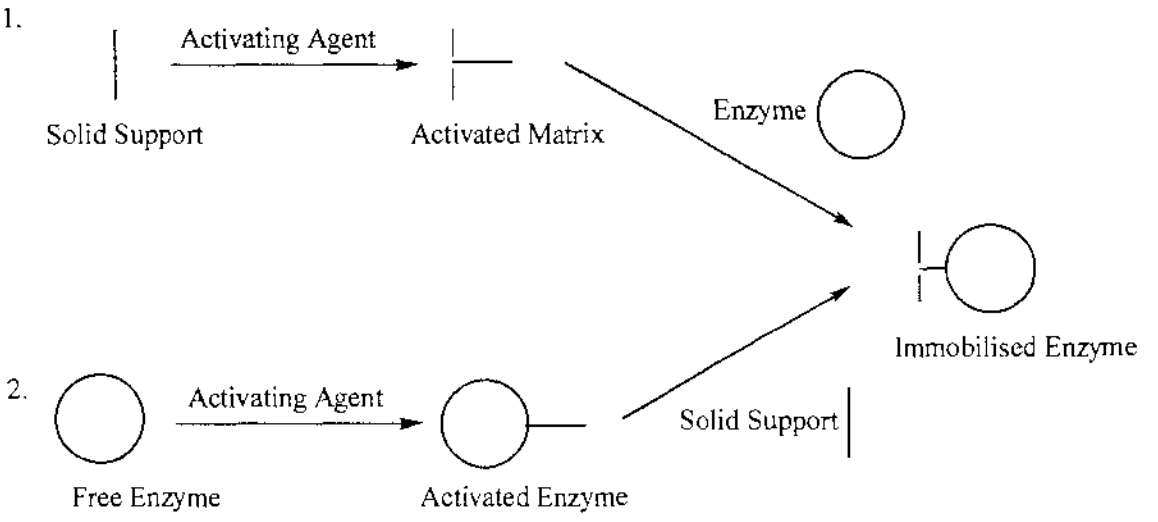


Figure 1.1 General Protocol For Covalent Binding (Rosevear, 1987)
1. The solid support can be activated and then enzyme added to react.
2. The enzyme itself is activated, then the solid support added.

Some heterogeneity in attachment of the enzyme due to different numbers of bonds between enzyme and support, and different sites of attachment on the enzyme is possible (Figure 1.2) (Pye and Chance, 1976). This can give several populations of enzyme with a variety of activity and stability. Relatively low substitution of active groups can minimise these problems by reducing the number of bonds individual enzymes can react with. Blocking the active site prior to coupling can stop bonding involving the active site.

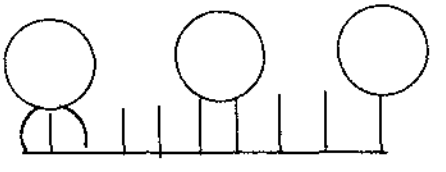


Figure 1.2 Heterogeneous Attachment

Activity can also be reduced by impairment of free movement of substrate to enzyme. Enzyme immobilisation requires the substrate to be transported from the bulk phase to the active site of the enzyme attached to a solid support and then product released back. Stability to unfavourable conditions such as temperature (Grusek *et al.* 1990, Hayashi and Ikada 1990) and pH extremes can be altered favourably due to the strong

bonding stabilising the enzyme structure. It is often difficult to regenerate supports used in this method after reaction with the chemicals used in immobilisation, so supports are generally not renewable. Optimal conditions for immobilisation can be difficult to find. The chemistries used in immobilisation can be expensive but this can be offset but the ability to reuse the enzyme. Disadvantages to immobilisation by covalent binding, such as lower initial activity and non-renewable supports, should be outweighed by the main advantages such as higher stability, high accessibility to macromolecular substrates and enzyme reusability.

The enzyme is attached to the support by either the side chain of one of several amino acids or the N-terminus or C-terminus. Side chains commonly used include lysine, cysteine, aspartic acid, glutamic acid and tyrosine (Table 1.2).

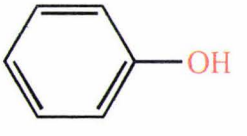
Residue	Structure
ε-amino of Lysine	—NH_2
Thiol of Cysteine	—SH
Carboxyl of Glutamic Acid or Aspartic Acid	—COOH
Phenolic of Tyrosine	

Table 1.3 Amino Acids Involved In Covalent Binding

Solid Supports

Numerous solid supports have been trialed for covalent binding. The list includes porous glass, ceramics, stainless steel, sand, charcoal, synthetic polymers and carbohydrates such as cellulose. Of the commercial matrices available the most common and widely applicable are the polysaccharides, agarose and cellulose, and their derivatives. Both contain hydroxyl groups available for activation. Matrices containing amide and ester groups are also commercially available. This group includes polyacrylamide, gelatine and nylon. Commercially available matrices such as polystyrene, porous glass and Celite have been used in enzyme immobilisation. Brick dust and chitin have also been trialed as solid supports. It appears almost anything may be used as a solid support for immobilisation of enzymes but several factors must be taken into consideration when selecting a solid support.

Factors In selecting solid supports (Cabral and Kennedy, 1991)

- Large Surface Area
- Permeability
- Hydrophilic Character
- Insolubility
- Chemical, Mechanical, and Thermal Stability
- High Rigidity
- Suitable Shape and Particle Size
- Resistance to Microbial Attack
- Regenerablity

Perloza™ Beaded Cellulose Resin

The support employed in this study is a beaded cellulose resin called Perloza™. Cellulose is a major structural polymer in plant material. It is a large polymer made up of β-D-glucopyranose units with β(1→4) linkages (Figure 1.2). The chains aggregate together to form stable, low-porosity structures which allow only groups on the polymer surface to be activated. Cellulose contains primary, secondary and vicinal diols, and so may be activated with a wide range of methods (Rosevear *et al,* 1987).

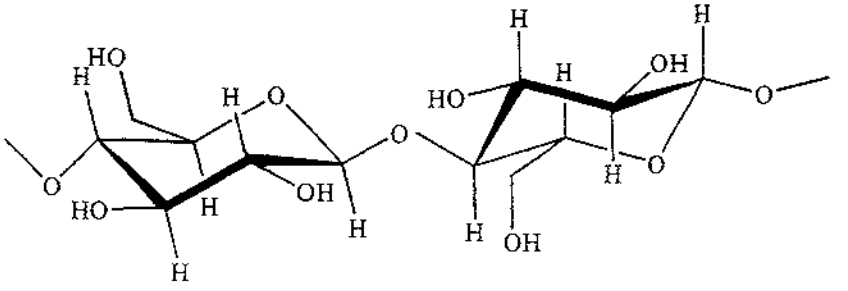


Figure 1.3 Cellulose Structure

Perloza™ is a beaded, non-covalently cross-linked regenerated cellulose resin produced by a "Thermal-Sol-Gel-Transition" (Stamberg, 1988) process. It is available in three particle diameter ranges fine (80-100μm), medium (100-250μm) and coarse (250-500μm), with three molecular weight exclusions 100, 250 and 500 kDa available in each. Fine grade Perloza™ was used throughout this study. Perloza™ has also been used for peptide synthesis (Englebretsen, 1992) and protein purification (Burton, 1995) at Massey University. It has a number of properties which make it suitable for enzyme immobilisation

Properties of Perloza™

- Good porosity to allow unimpaired movement of large molecules.
- An ample supply of chemical groups which may be activated.
- Hydrophilic, therefore little, non-specific, hydrophobic interaction with proteins and the carbohydrate backbone.
- Uniform, spherical shape of individual particles, allowing an even distribution of enzyme.
- Mechanically and chemically stable.
- Relatively cheap and available

Activation Methods for Covalent Bonding

The presence of free amino, carboxylic, phenolic and thiol groups on the surface of proteins allows them to be attached with a wide variety of activation chemistries. A small selection of the chemistries available for activation is shown in Table 1.4.

Glutaraldehyde, epichlorohydrin, carbodiimidazole and periodate are among of the more useful reagents due to their low toxicity and stable bonds. Cyanogen Bromide (CNBr) has also been widely used for enzyme immobilisation since it was first described by Axén and co-workers 1967. This is despite extreme toxicity, formation of three types of bond between ligand and substrate, inclusion of some ion exchange interactions and ligand leakage (Gerartz, 1990).

Coupling Reaction	Description
Diazotization	Diazo linkage between protein mainly His and Tyr residues and aryldiazonium groups on carrier.
Amide Bond Formation	Amide (peptide) bond formed between nucleophilic groups on protein (amino, hydroxy and thiol) and carrier.
Alkylation and Arylation	Alkylation of amino, phenolic or thiol groups on protein with active halides, oxirane, vinylsulfonyl or vinylketo groups on the support.
Schiff's Base Formation	Formation of a Schiff's base (aldimine) link between free amino groups on protein and carbonyl groups on support.
Ugi Reaction	Formation of an N-substituted amide between protein and carrier.
Amidination Reactions	Imidoesters on carrier react with protein amino groups to give an amidine linkage.
Thiol-disulphide Interchange	Formation of a disulphide linkage between thiol groups on support and protein.
Mercury-enzyme Interactions	Thiol groups on protein react with mercury derivatives carriers (can also be classed as physical adsorption)
γ-Irradiation Induced Coupling	Radicals formed on protein and carrier by γ-irradiation combine to form a covalent bond.

Table 1.4 A Selection of Activation Chemistries

Activation Methods Studied

1,1'-Carbonyldiimidazole (CDI) Activation

In this study several methods of activation were trialed on Perloza™. Initial experiments focused on activation by CDI, a method used previously for attachment of smaller ligands and enzymes (Hearn 1987, Burton *et al* 1991). An amide bond is formed between the enzyme and the support. CDI is reacted with hydroxyl groups present on Perloza™ to form a reactive imidazole carbamate which has good chemical stability at pH 4-10. A stable carbamate linkage can then be formed between the activated matrix and an amine containing ligand, which can be either an enzyme or spacer arm (Figure 1.4a) (Hearn, 1987). CDI is moisture sensitive so reactions must be carried out in anhydrous solvents such as acetone and dioxan. Enzymes may be attached directly to the resin or through a spacer arm such as 6-amino caproic acid (Burton *et al* 1991). The spacer arm must then be activated for enzyme immobilisation. Attachment of enzyme to a CDI activated matrix is carried out at pH 8.5-11 which may not suit some enzymes. CDI activation gives a linkage with no charge, which minimises non-specific ionic binding and produces a relatively stable linkage that reduces ligand leakage. However some leakage can occur at pH extremes.

Choropropanediol/Periodate Activation

A second activation method trialed was attachment via an aldehyde moiety (Burton *et al* 1996). Periodate oxidation is a common way to form aldehydes from the alcohol groups on cellulose; this however weakens the matrix. The problem can be avoided by first attaching diol groups which provide a more accessible and so more reactive group for modification. Chloropropanediol is used to attach the diol group to Perloza™. The diol groups are then oxidised to form a pendant aldehyde with release of formaldehyde. Reaction of the pendant aldehyde with amine groups of the enzyme results in immobilisation of the enzyme via a Schiff's base, which is then hydrogenated by sodium cyanoborohydride to give a strong covalent linkage (Figure 1.4b). Sodium cyanoborohydride was chosen over sodium borohydride for this reaction as it is more specific for the aldimine linkage.

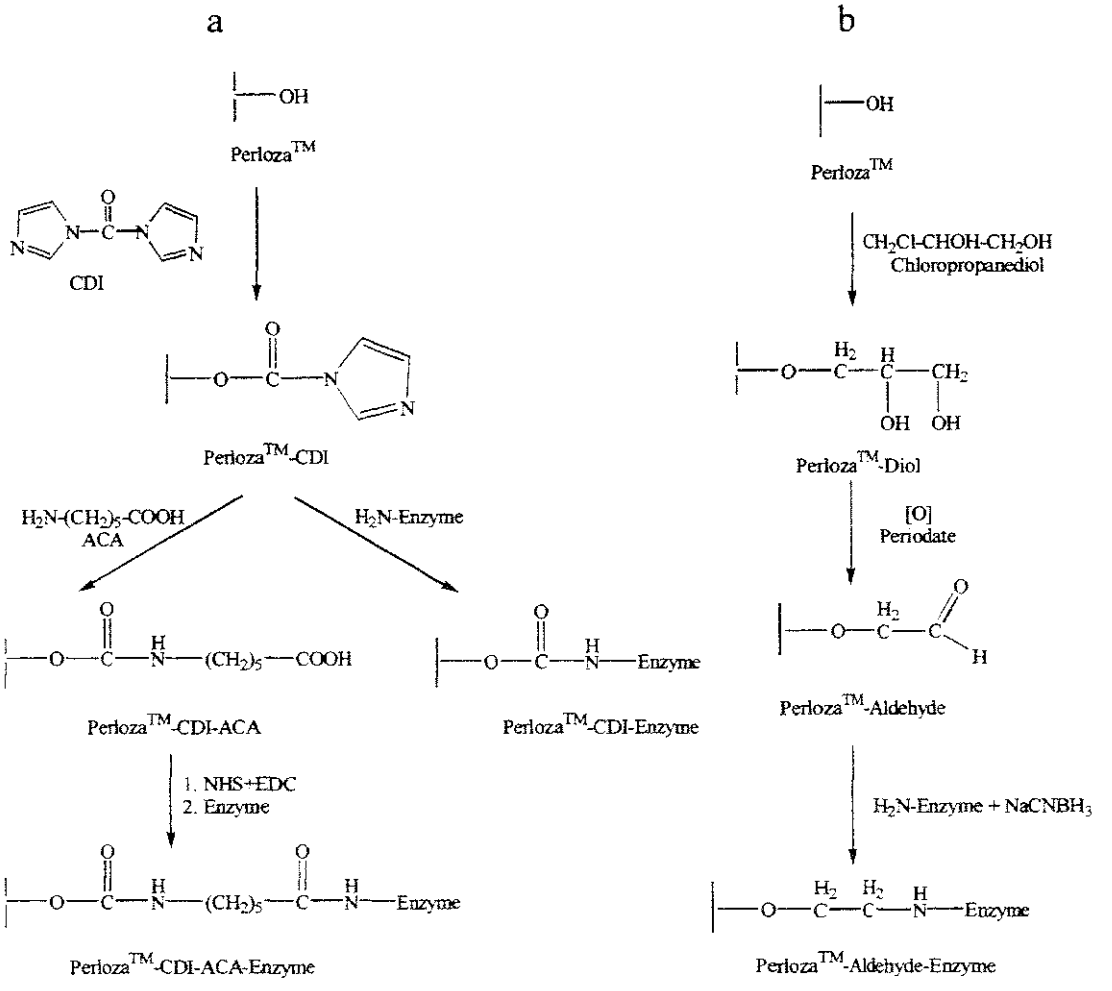


Figure 1.4 Attachment Chemistries Used a) CDI Based Attachment

b) Diol/Aldehyde Based Attachment

Investigations in the Immobilisation of Enzymes to Perloza™

The enzymes used in this study were chosen for their availability, relative cost, ease of assay and cost of assay (Table 1.5). A variety of a enzymes was also deemed to be desirable, therefore two proteolytic enzymes, trypsin and chymotrypsin were studied in detail and three other enzymes were attached via all three methods and initial activity assayed.

The studies reported in this thesis aim to give an indication that a variety of enzymes can be successfully immobilised on Perloza™ using well-reported chemistries and a novel method. The enzymes used in the study have been immobilised previously and

used in industry therefore applications for such preparations are available for the enzymes studied and others using the chemistries studied.

The studies reported in chapters 3 and 4 concentrated on the effect immobilisation had on the activity of trypsin and chymotrypsin. The studies in chapter 5 concentrated on extending the number of enzymes immobilised on Perloza™ by the three methods studied and comparing their initial activity with that of free enzyme.

Enzymes	Classification	Molecular Weight
Trypsin	Serine Protease	23,000
Chymotrypsin	Serine Protease	25,000
Amylase	Glycolase	48,000
Alcohol Dehydrogenase	Dehydrogenase	150,000
Horseradish Peroxidase	Peroxidase	44,000

Table 1.5 Enzymes Used in this Study

Analysis

The activation of Perloza™ by each method was followed by titration. Protein substitution was measured by the bicichinonic acid assay. The activity of trypsin and chymotrypsin was also compared with free enzyme by reaction with a "natural" substrate (a synthetic peptide), and this reaction was followed by HPLC. Enzyme activity for trypsin, chymotrypsin and amylase was measured with chromogenic substrates specific for each enzyme. Activity assays were also used to study the specific activity of alcohol dehydrogenase and horseradish peroxidase. Specific

activity was calculated to compare the effectiveness of immobilisation methods from the activity assays.

Specific Activity

Specific Activity can be defined as the catalytic activity of the enzyme related to its protein content. It is usually expressed as units per miligram. The Enzyme Commission of the International Union of Biochemistry has defined the *International Unit* U as "the activity of an enzyme which, under optimised standard conditions, catalyses the conversion of 1 μmol of substrate per minute". It is used in this study to compare the activity of immobilised enzyme between immobilisation methods and to follow the inactivation of enzyme after heating.

CHAPTER TWO

METHODS AND MATERIALS

Reagents and Equipment

Perloza™ fine grade bead cellulose was from Tessek Ltd of Prague, Czechoslovakia. Trypsin type IX (BAEE 14 600), α-chymotrypsin type II, benzoyl-DL-arginine-ρ-nitroanilide HCl (BAPNA), N-glutaryl-L-phenylalanine ρ-nitroanilide (GAPNA), 6-aminocaproic acid (ACA), 1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), bicinchoninic acid (BCA), horseradish peroxidase (HRP) and alcohol dehydrogenase (ADH) were from Sigma Chemical Co., St Louis, MO, USA; 1,1'-carbonyldiimidazole (CDI) was from Janssen Chimica Geel, Belgium; Sodium cyanoborohydride and chloropropanediol were from Aldrich Chemical Company, Inc., Steinheim, Germany; Ceralpha substrate was from Deltagen Australia Pty. Ltd., Boronia, VIC., Australia; Convol NaOH, barbitone, boric acid, acetonitrile and calcium chloride were from BDH, Dorset, England. Sodium dihydrogen phosphate and copper (II) sulphate pentahydrate were from Riedel-de-Haën, Selze, Germany; Amylase was from Genencor International Inc., Palo Alto, CA, USA and purified by Dr S. Burton, Department of Chemistry, Massey University, New Zealand; NAD^+ was from Boehringer Mannheim GmbH, Germany; Sodium Periodate, pyrogallol, dioxan and sodium azide were from Ajax Chemicals Ltd., Auburn, NSW, Australia; 4-hydroxymethylphenoxymethyl-copolystyrene-1% divinylbenzene (HMP) resin was from Applied Biosystems, Australia. Water was MilliQ grade

Analytical HPLC was carried out on an 250mm x 4.6mm Econosphere C18 5μm column and preparative HPLC purification runs were carried out on a 250mm x 10mm Synchroprep C18 column. Both columns were from Altech. A Radiometer ETS822 autotitrator was used for resin titrations. A Hitachi U-1100 spectrophotometer was used for absorbance measurements. The HPLC system used was a Waters Associates system comprised of a model 680 automated gradient controller, 2 model 510 pumps and a model 441 absorbance detector. Reactions were

mixed by rotation on a Cole-Parmer Roto-Torque or shaken on an Ika-Vibra-mix. Activity assays and BCA assays were incubated in a Julabo SW-20C water bath. Incubations at 60°C were heated in a Grant water bath. An Applied Biosystems 431A Peptide Synthesiser was used for peptide synthesis. A SCIEX 300 ES-MS-MS Triple Quad Mass Spectrometer was used for mass analysis.

Resin substitutions after activation are shown as mmol of activated groups per g of dry resin after the resin used for titration was dried at 100°C for 1 hour. Enzyme substitutions are shown as mg of protein per gram of wet resin after excess water is removed from the resin in a sintered glass funnel with vacuum

Perloza™ Activation with 1,1'-Carbonyldiimidazole (CDI)

Solvent Exchange

Perloza™ MT 200 fine was washed thoroughly with distilled water. The resin was then solvent exchanged to dioxan with 33%, 66% and 100% dioxan using a column mode of solvent exchange. Perloza™ was covered with Whatman filter paper, allowing solvents to be poured onto the column without disturbing the surface. Washes were allowed to filter slowly through the resin for thorough equilibration (Burton, 1995).

CDI Reaction

CDI (0.06g per dry gram Perloza™) was added to anhydrous resin. The mixture was shaken/rototorqued for 1 hour at room temperature. For direct enzyme attachment the resin was solvent exchanged back to water, washed thoroughly with distilled water and a sample taken for titration. For 6-aminocaproic acid attachment, resin was left in dioxan and a sample taken and washed back to water for titration (Burton, 1995).

Enzyme Attachment to Perloza™-CDI

Trypsin (10mg/ml) was dissolved in cold 50mM barbitone, 200mM $CaCl_2$ pH 9.0, chymotrypsin (10mg/ml) in cold 50mM barbitone pH 9.0 and amylase in 50mM borate pH 9.0 for their respective attachments. Two resin volumes of enzyme mixture

were added to the resin and shaken for 1 hour at room temperature. The resin was then washed with buffer or 0.15M NaCl (Burton, 1996). The protein concentration was determined by BCA assay (described below) and reported as mg of protein per g of wet resin.

6-Aminocaproic Acid (ACA) Attachment to Perloza™-CDI

A 5 molar excess of ACA was weighed into a small beaker and dissolved in a minimum volume of 5M NaOH. The pH was adjusted to 11.3 and the mixture added to dioxan wet Perloza™-CDI and the mixture was shaken overnight at room temperature. The resin was then solvent exchanged back to water and washed extensively with distilled water. A small sample (~1g wet weight) was taken for titration (Burton, 1995).

Enzyme Attachment to Perloza™-CDI-ACA

A 20 fold excess of N-hydroxysuccinimide (NHS) and 1-ethyl-3-(dimethylamino-propyl) carbodiimide hydrochloride (EDC) over activatable groups was added to dioxan wet Perloza™-CDI-ACA and the mixture shaken at room temperature for 1 hour. The resin was washed with dioxan and solvent exchanged back to water.

Trypsin (10mg/ml) was dissolved in cold 50mM barbitone, 200mM $CaCl_2$ pH 9.0, chymotrypsin (10mg/ml) in cold 50mM barbitone pH 9.0 and amylase in 50mM borate pH 9.0, for respective couplings. Two resin volumes of enzyme mixture were added to the resin and the mixture shaken at room temperature for 1 hour. The resin was then washed with buffer or 0.15M NaCl (Burton, 1996). Protein concentration was determined by BCA assay and reported as mg of protein per g of wet resin.

Diol Activation

Chloropropanediol (4ml) and 3M NaOH (20ml) were added to water wet Perloza™ (20g wet) and shaken overnight at room temperature. The resin was then washed with distilled water (Burton *et al*, 1996).

Periodate Oxidation

0.05M periodate (12ml per 10g wet Perloza™) was added to Perloza™ and shaken overnight at room temperature to oxidise the diol groups to aldehyde. Aldehyde substitution was determined by titration and expressed as mmol aldehyde groups per dry g of resin (Burton *et al*, 1996).

Titration Methods

Imidazole Titration

This process requires the hydrolysis of the labile imidazole groups from the resin. For the hydrolysis 10ml of 0.1M NaOH was added to the resin sample (~1g wet weight) in a titration vessel. The mixture was left overnight to allow complete hydrolysis. The mixture was acidified with 1.5ml of 1M HCl and flushed with nitrogen. The pH was adjusted to 5 with 1M NaOH and titrated to 8.5 with 0.1M NaOH. The resin sample was washed into a pre-weighed funnel and oven-dried for 1.5 hours at 110°C. The sample was allowed to cool to room temperature under vacuum in a dessicator and the dry resin weight calculated. The concentration of titratable groups was expressed as mMoles/g dry resin (Burton, 1995).

ACA Titration

The resin was washed slowly with 5 funnel volumes of 0.1M HCl and then washed thoroughly with distilled water until flowthrough pH matched water pH. The resin was then washed into a titration vessel and titrated with 0.1M NaOH to pH 8 in the presence of 0.4mol NaCl. The sample was then rinsed and dried as for imidazole titration, to allow estimation of titratable groups in mMoles/g dry resin.

Titration of Matrix Aldehyde Groups

Girard's Reagent T (0.1g) was added to a Perloza™ sample (1.2g wet resin) and the pH was then adjusted to 8 and left to react overnight. The sample was then treated with sodium borohydride (50mg) at pH 9 for reduction. The sample was washed out with water and 0.1 HCl. The sample was then washed with water again and 0.1M

NaOH. The sample was then washed with water until the flowthrough matched the water pH. Titration was carried out with 0.1M HCl to pH 4 and substitution expressed as mmol/g dry resin (Burton, 1995).

Enzyme Attachment to Perloza™-Diol/Aldehyde

Enzymes were dissolved in 100mM phosphate pH 6 (10mg/ml), added to aldehyde activated Perloza™ (2ml per 1g wet resin) in the presence of sodium cyanoborohydride (10mg per 1g wet resin) and reacted at room temperature for 1 hour. Protein concentration was determined by BCA assay and reported as mg of protein per g of wet resin (Burton *et al*, 1996).

Bicinchoninic Acid (BCA) Protein Concentration Determination

50 parts bicinchoninic acid were mixed with 1 part copper (II) sulphate pentahydrate 4% solution to make the BCA (protein determination) reagent. 1mg/ml of trypsin, chymotrypsin or amylase was used to make a standard curve of 0-100μg protein (0.1ml). BCA reagent (2ml) was added to each of the tubes which were incubated for 30 minutes at 37°C in a shaking water bath. Absorbance was measured at 562nm against a water blank. Final absorbance was calculated by subtracting a zero value solution (0.1ml water per 2 ml protein determination reagent incubated at 37°C for 30 minutes) to produce a standard curve of absorbance versus μg protein (Stich, 1990).

Protein determination reagent (2ml) was added to resin samples (0.1g wet weight) and incubated as for the standard curve. Protein concentration was determined from the standard curve and expressed as mg protein per gram wet resin.

Enzyme Activity Assays

Trypsin BAPNA Assay

Resin samples (25μg) were weighed accurately into vials and 100mM Tris-HCl pH 8.0 (1.4ml) added. 1mg/ml BAPNA solution (1.4ml) was added and samples

incubated at 25°C for various times up to 20 minutes. Reactions were halted by the addition of 30% v/v acetic acid aqueous solution (2ml). Absorbance was measured at 410nm (Huckel *et al*, 1996).

Chymotrypsin GAPNA Assay

Resin samples (50μg) were weighed accurately into vials and 50mM Tris-HCl pH 9 (700μl) added. 2.5mM GAPNA solution (700μ) was added and the mixture incubated at 25°C in a shaking water bath for various times up to 40 minutes. Reactions were halted by the addition of 30% v/v acetic acid. Absorbance was measured at 410nm (Huckel *et al*, 1996).

Horsradish Peroxidase (HRP) Activity Assay

0.1M potassium phosphate (0.32ml), 0.147M H_2O_2 (0.16ml), 5% w/v pyrogallol solution (0.32ml) and H_2O (2.10ml) were added to a cuvette inverted and zeroed on the spectrophotometer at 420nm. Enzyme was then added, the cuvette mixed, left for 15 seconds and then the reaction followed for 60 seconds (Walters, 1986).

α-Amylase Activity Assay

p-Nitrophenyl maltoheptaoside (BPNPG-7), Cerapha substrate, was diluted 4-fold with 50mM malate buffer, 5mM $CaCl_2$, 0.002% Tween 20, pH 6.7. α-Amylase sample (100μl) was added to the substrate-buffer mixture (700μl) in a cuvette and inverted. The absorbance was then monitored at 410nm for 2 minutes after an initial lag period of 75 seconds (Burton, 1995).

Alcohol Dehydrogenase (ADH) Activity Assay

The spectrophotometer was first zeroed on 0.6M Tris/lysine buffer (2.6ml), 0.006M NAD^+ (0.2ml) and 0.15M ethanol (0.2ml). To measure activity 0.6M Tris/lysine buffer (2.4ml), 0.006M NAD^+ (0.2ml) and enzyme were added to a cuvette and inverted. 0.15M ethanol (0.2ml) was then added and the reaction followed for 1 minute at 340nm on the uv spectrophotometer (Cornell and Veech, 1983).

Synthetic Peptide Synthesis and Purification

Synthesis

Peptide synthesis was carried out using Fastmoc™ chemistry on a standard ABI HMP resin. Peptide was cleaved from the resin using 95% TFA (9.5ml), water (0.25ml) and 1,2-ethanedithiol (0.25ml) for 1.5 hours at room temperature. Scavengers were removed by washing with diethyl ether. Samples were freeze-dried in preparation for purification (Applied Biosystems Inc, 1990).

Purification

Synthetic peptide was purified on an HPLC. Buffer A was 98% water, 2% acetonitrile, 0.1% TFA and buffer B was 98% acetonitrile, 2% water, 0.1% TFA. All samples were filtered through a 0.2µm Millipore membrane in a Swinny filter. A linear gradient was run at 1% per 4 minutes up to 20% B. Fractions were collected at 2 minute intervals across the main peak, then analysed by HPLC and pure samples pooled and freeze dried.

Trypsin and Chymotrypsin Digestion of Synthetic Peptide

Synthetic peptide (1mg) was dissolved in 0.1M ammonium bicarbonate solution (1ml). An enzyme sample (~0.01mg) in ammonium bicarbonate solution (0.5ml) was added to digest the peptide and the mixture shaken. A further addition of enzyme was made after 2 hours. Digestion was halted after overnight incubation by the addition of formic acid to lower the pH to 2-3. Samples were then separated on HPLC and the peaks collected and analysed by amino acid analysis and mass spectrometry (Fisher, 1997).

Amino Acid Analysis Preparation

Samples from the HPLC were hydrolysed by 6M HCl and 0.1% phenol (1ml per mg sample or 200µl) under vacuum at 110°C overnight. The sample was then dried down in a dessicator. Amino acid analysis (AAA), was performed by Debbie Frumau, Institute of Molecular Biosciences, Massey University and Mr N. Greenhill,

Malaghan Institute, Wellington School of Medicine, University of Otago. The comparative amounts of each amino acid present were determined relative to alanine

.

Mass Spectroscopy

Mass Spectrometry was performed by Ms Jo Mudford, Institute of Molecular Biosciences, Massey University.

CHAPTER THREE

TRYPSIN

Introduction

Trypsin is a major proteolytic enzyme found in the pancreas (Walsh, 1970). It is involved in the activation of all serine proteases from their zymogen precursors. Trypsin cleaves at the carboxamide bond of lysine and arginine (Cunningham, 1965). Activation *in vivo* is by limited autolysis from a zymogen precursor. Trypsin is involved in the activation of other zymogens by limited proteolysis of their precursors. It is a serine protease, a family that includes thrombin, elastase, subtilisin and chymotrypsin. Each member of the family contains a serine in the active site. Trypsin has been used as a model for other similar enzymes (Kasai, 1992).

The porcine trypsin used in this study comprised of 219 amino acids. It had 10 lysine residues, potential attachment sites using CDI, ACA and diol activation methods (Travis & Liener, 1965; Walsh, 1970). Resin substitution levels were kept relatively low to reduce the possibility of attachment at multiple sites.

Trypsin has been immobilised previously using several methods, including covalent binding (Huckel *et al*, 1996), adsorption to carbon (Mukherjea *et al*, 1980) and microencapsulation (Ezpeleta *et al*, 1997). Its activity in various immobilisation systems has been studied extensively and a large body of literature is available.

Resin substitutions after activation are given as mmol of activated groups per g of dry resin after the resin used for titration was dried at 100°C for 1 hour. Enzyme substitutions are shown as mg of protein per gram of wet resin after excess water had been removed from the resin in a sintered glass funnel with vacuum.

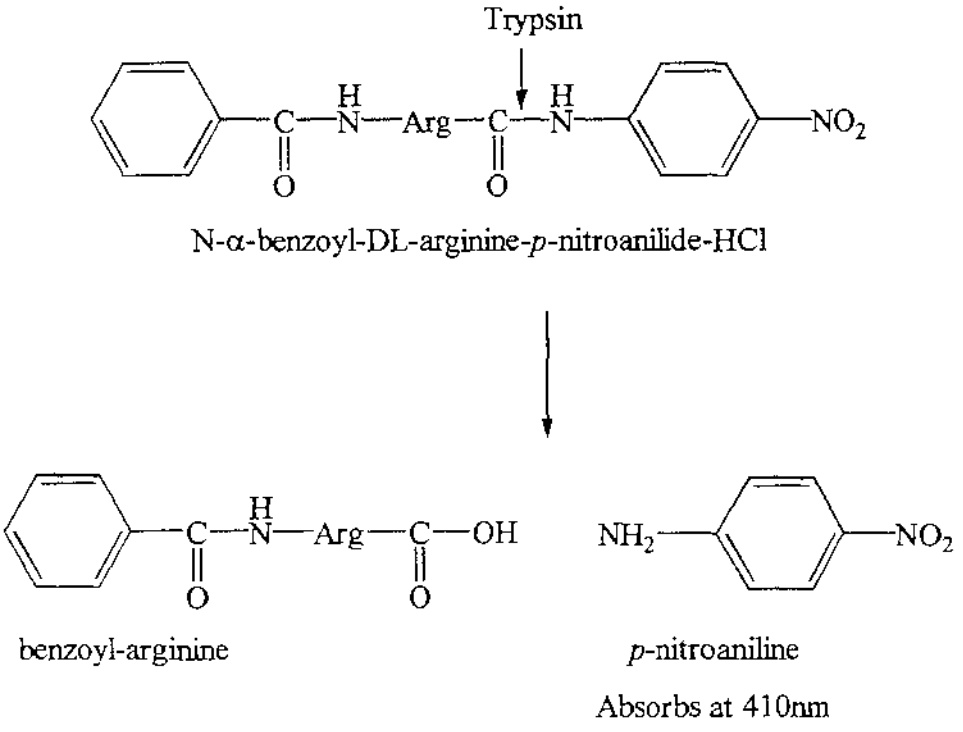


Figure 3.1a BAPNA Assay for Trypsin Activity
Trypsin cleaves the carboxamide bond to produce *p*-nitroaniline which absorbs at 410nm

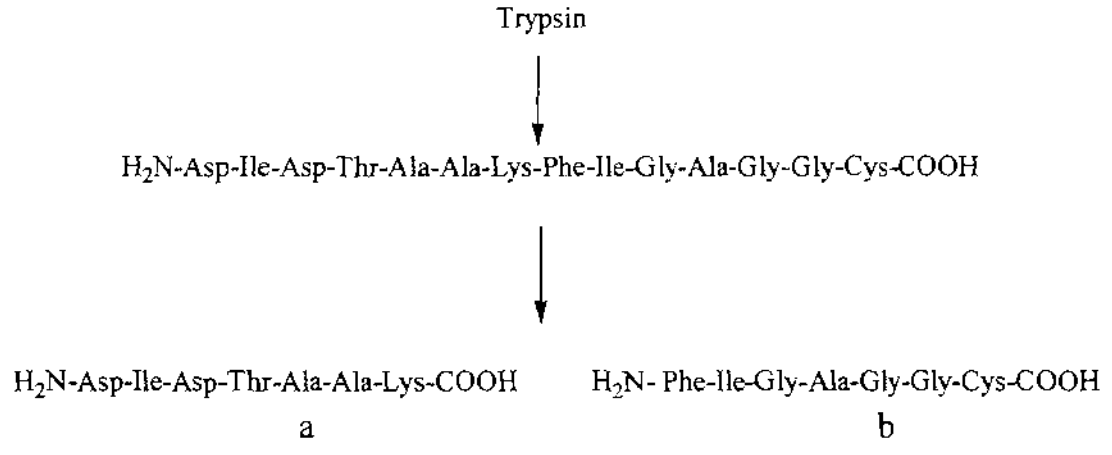


Figure 3.1b Trypsinolysis of "Real" Substrate
Trypsin cleaves the carboxyamide bond between lysine and phenylalanine to give two peptides a and b separable by HPLC.

Results and Discussion

Proteolytic activity of trypsin was determined from the BAPNA assay. The enzyme acts on the artificial chromogenic substrate by cleaving the carboxyamide bond (Figure 3.1a). Digests of a synthetic peptide were also performed as an example of "real" activity (Figure 3.1b). A synthetic peptide was selected over a larger protein because it would result in a simpler HPLC trace and give fewer peaks for analysis by HPLC and mass spectrometry. The synthetic peptide (Figure 3.1b) was a 14mer with a molecular weight of 1338.5 and one lysine residue at position 7. Digestion with trypsin would therefore result in two 7mer peptides able to be separated on HPLC and distinguished by amino acid analysis (AAA) and mass spectrometry. Peptide **a** has a molecular weight of 732.9 Da and peptide **b** had a molecular weight of 624.0 Da.

Activation of Perloza™

Due to the successful results obtained by Walters (1986) using CDI activation on Sepharose, it was decided to start initial studies using the same chemistry for immobilisation of trypsin with Perloza™. Substitution levels of CDI were between 0.25 and 0.28 mmol/g dry resin. This range was lower than used previously for immobilisation of amino acyl spacer arms (Burton *et al*, 1991). The substitution levels in this trypsin study were designed to minimise the possibility of multiple attachment, i.e. attachment at several lysine sites on the enzyme. Substitution levels were monitored by titration of imidazole groups. CDI activation levels for immobilisation via an ACA spacer arm were similar to those for direct immobilisation. ACA substitutions for initial experiments were between 0.210 and 0.282 mmol/g dry resin. Activity assays in the heat studies of enzyme attached at this level of substitution were inconsistent. Enzyme attachment at a lower level of ACA substitution 0.137mmol/g appeared to give more consistent results in the heat studies. This was possibly due to a reduced chance of multiple attachments occurring. Aldehyde substitutions were between 0.270 and 0.295mmol/g dry occurring.

Incubation Time (min)	Substitution (mg/g wet resin)
30	2.98
45	3.37
60	4.19
90	4.38
120	4.93

Table 3.1 Trypsin Substitution Levels on CDI Activated Perloza™
CDI Substitution = 0.24 mmol/g dry resin

Enzyme Attachments

As a proteolytic enzyme trypsin rapidly undergoes autolysis at pH greater than 6.0. Autolysis can be limited by the addition of Ca^{+} or adjustment to low pHs (Green and Neurath, 1953). Calcium chloride ($CaCl_2$) was used in the barbitone buffer pH 9.0 for immobilisations using CDI activated resins to limit autolysis as the attachment buffer pH is near the optimum pH 8.0 for trypsin. In order to find the optimum time for attachment initial attachments directly to CDI activated resins using small samples of resin (~1g wet weight) were for various times between 30 minutes and 2 hours (Table 3.1). Incubation times of 1 hour were selected for larger scale attachments (10g wet weight or more), which gave a good level of substitution and minimised the time available for trypsin autolysis. Trypsin substitutions for larger scale preparations were between 2.61 and 3.00 mg/g wet resin. Smaller scale attachments gave a higher substitution than larger scale. Enzyme attachments to resins with high ACA substitution enzyme levels were between 7.25-7.76 mg/g wet resin. Resin with a lower ACA substitution had an enzyme level of 2.94 mg/g wet resin. To find the optimum time for immobilisation aldehyde resins were incubated with trypsin for either 1 hour or 16 hours which was the time used for immobilisation of smaller ligands (Burton *et al*, 1996). Substitution levels found were 7.25 mg/g wet resin for 1 hour incubation and 7.1 mg/g wet resin for 16 hour incubation. Enzyme substitution on aldehyde resin used in the heat study was 7.72 mg/g wet resin. Attachment to aldehyde resin was carried out without $CaCl_2$ due to its insolubility in phosphate buffers. The pH of those buffers

was 6.0, lower than the optimum pH of trypsin at 8.0, therefore trypsin autolysis would be minimised.

BAPNA Assay

Initial BAPNA assays were based as much as possible on methods from Walters (1986), however few details were available on the exact assay method. The buffer used was 100mM Tris.HCl pH 7.8 with and without $CaCl_2$. Samples of Trypsin-Perloza™ conjugates were weighed into vials and buffer was added to give suspensions of varying concentration. A sample of this suspension was then pipetted into vials and BPNA added. To terminate the reaction samples were filtered from the supernatant. Results were erratic and difficult to reproduce due to difficulties in delivering reproducible amounts of immobilised trypsin.

Control activity and BCA assays were done with each resin without enzyme attached.

In these initial activity studies 0.05ml of 80 mg/ml trypsin in buffer was measured by pipette into reaction mixtures. The assay was then altered to be similar to the method used by Huckel et al 1996. Volumes used in the assay were altered to give better mixing. It was decided not to use $CaCl_2$ in the assay buffer from Hucklel et al 1996 so any stabilisation noted would be directly due to viable immobilised trypsin.

Heat Study at 60 ℃

Initial results with Perloza™-CDI-Trypsin using Walters (1986) assay methods indicated trypsin retained between 14.8 and 29.5% of the initial activity after 7 days at 60°C (Table 3.2). In most experiments there was a sharp drop to 50% of initial activity from the time zero until 3 hours, after which the activity loss levelled off at a lower percentage. Results from initial experiments showed significant variation between duplicates and were difficult to replicate consistently. Figure 3.2a shows the general shape of the activity vs. time graphs obtained from these preliminary experiments. These results were from several different batches of resin with variations in the assay mixture, therefore average percentages are shown rather than specific activities. Initial studies on Perloza™-CDI-ACA-Trypsin using similar buffer mixtures

and assay methods also gave variable results. Total activity appeared to drop to 39% of initial activity after 7days at 60°C (Figure 3.2b, Table 3.2).

Preliminary experiments on Perloza™-CDI-Trypsin were done with $CaCl_2$ in the assay buffer on Perloza™-CDI-Trypsin (Table 3.3, Figure 3.2c). Immobilised trypsin in analysed in buffer without $CaCl_2$ retained a slightly higher percentage activity after 24 hours than with $CaCl_2$. Again results show variation, but indications were that there was no significant difference in trypsin activity due to the presence of $CaCl_2$ therefore it was decided not to use it in the assay buffer.

Time at 60°C (min)	Perloza™-CDI-Trypsin (%)	Perloza™-CDI-ACA-Trypsin (%)
0	100	100
10	82.1	81.5
20	92.3	78.0
30	84.6	77.0
60	76.9	71.5
120	64.1	73.0
180	61.5	65.5
360	49.2	64.5
720	37.4	61.5
1440	20.5	67.5
2880	27.2	62.0
4320	20.0	62.5
5760	16.9	51.5
7200	16.4	47.0
8640	15.2	41.0
10080	14.8	39.0

Table 3.2 Preliminary Heat Studies of Perloza™-CDI-Trypsin and Perloza™-CDI-ACA-Trypsin at 60°C. Results Shown as a Percentage of Original Activity. Plotted in Figure 3.2 a and b respectively.

Time at 60°C (min)	Percentage Retained Activity With $CaCl_2$	Percentage Retained Activity Without $CaCl_2$
0	100	100
10	79.2	95
20	83.3	90
30	95.8	85
60	70.8	80
120	66.7	60
180	54.2	65
360	41.7	50
720	32.1	45
1440	25.0	35

Table 3.3 Heat Studies Comparing Buffer With or Without $CaCl_2$ up to 1 Day (1440 minutes). Plotted in Figure 3.2 c.

Free trypsin had a specific activity of 0.351 μmol min^{-1} mg^{-1} and lost 88.3% activity after 10minutes at 60°C. Trypsin directly attached to Perloza with CDI was incubated at 60°C for times ranging from 10 minutes to 2 days. After 2 days the activity reached a level at which it could not be reliably measured (Figure 3.3, Table 3.4). Initial specific activity was 0.251μmol min^{-1} mg^{-1}. Activity dropped sharply until 6 hours (360min), but then it levelled out. Trypsin immobilised via an ACA spacer arm retained 11.0% of its original activity after heating at 60 degrees for 1 week. Specific activity for trypsin immobilised with an ACA spacer arm started higher than direct attachment at 0.345 μmol min^{-1} mg^{-1}, a similar level to the 0.35μmol min^{-1} mg^{-1} of free trypsin. Activity dropped quickly until 2 hours and then dropped more slowly. Trypsin immobilised on aldehyde resins had a much lower starting specific activity at 0.128 μmol min^{-1} mg^{-1} compared with the two CDI-based resins, but lost less activity.

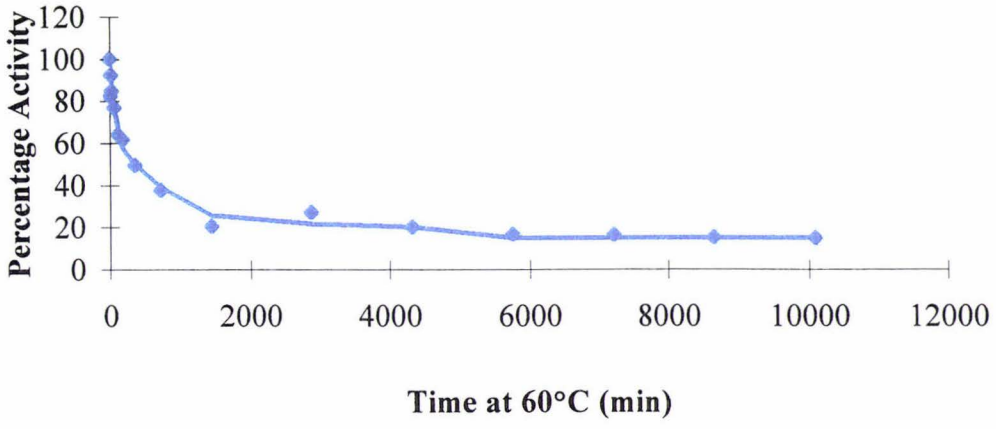


Figure 3.2a Composite Results From Preliminary Perloza™-CDI-Trypsin Heat Study At 60°C.

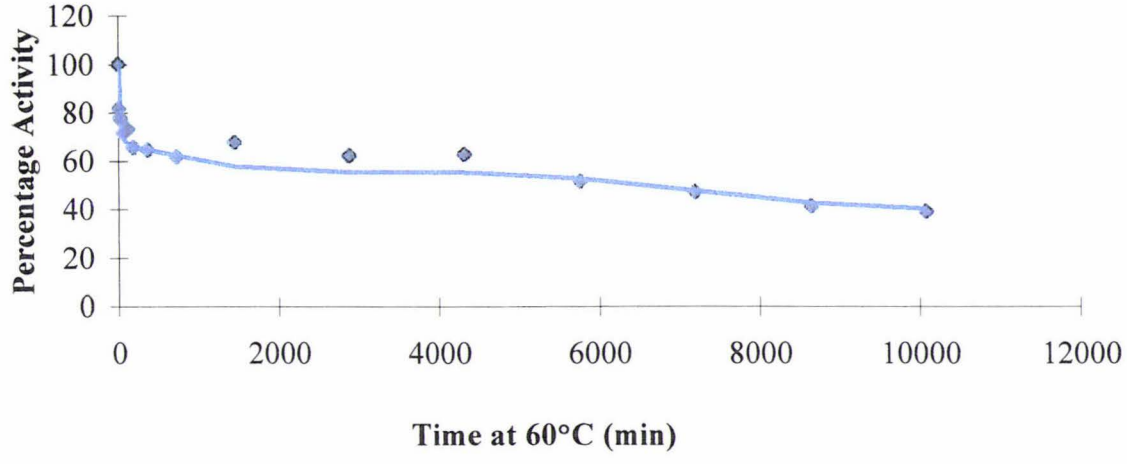


Figure 3.2b Composite Results From Preliminary Perloza™-CDI-ACA-Trypsin Heat Study At 60°C.

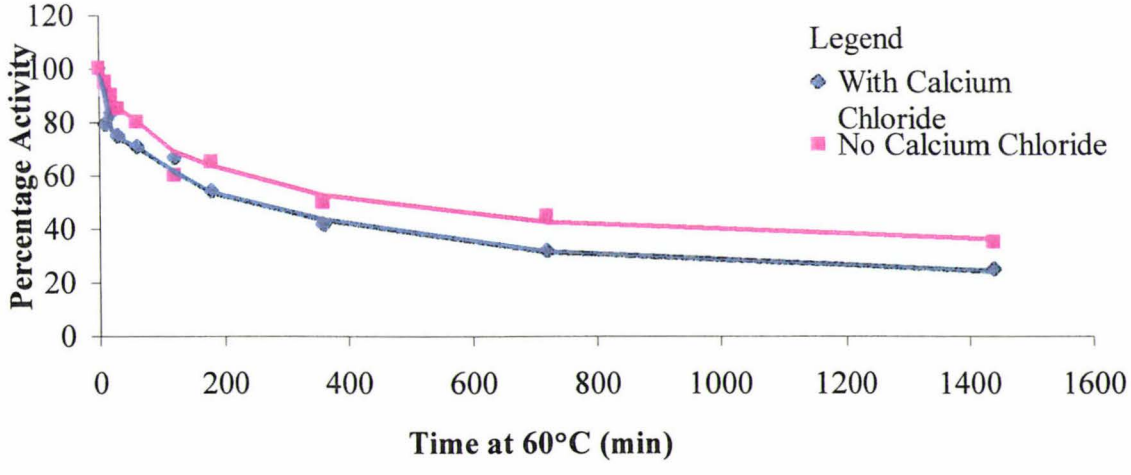


Figure 3.2c Heat Study at 60°C, a Comparison With and Without $CaCl_2$.

Time at 60°C (min)	Perloza™-CDI-Trypsin (μmol min^{-1} mg^{-1})	Perloza™-CDI-ACA-Trypsin (μmol min^{-1} mg^{-1})	Perloza-Aldehyde-Trypsin (μmol min^{-1} mg^{-1})
0	0.251 (100)	0.345 (100)	0.128 (100)
10	0.225 (89.8)	0.301 (87.2)	0.127 (99.2)
20	0.189 (74.5)	0.280 (81.2)	0.118 (92.2)
30	0.177 (70.8)	0.269 (78.0)	0.119 (93.0)
60	0.145 (58.1)	0.251 (72.8)	0.114 (89.1)
120	0.095 (37.9)	0.244 (69.6)	0.106 (82.8)
180	0.089 (35.4)	0.249 (62.0)	0.100 (78.1)
360	0.056 (22.2)	0.240 (55.1)	0.095 (74.2)
720	0.051 (20.5)	0.214 (37.7)	0.094 (73.8)
1440#	0.041 (16.3)	0.190 (30.4)	0.095 (74.2)
2880	0.042 (16.7)	0.130 (22.3)	0.094 (73.8)
4320		0.105 (17.7)	0.092 (71.9)
5760		0.077 (13.0)	0.089 (69.5)
7200		0.061 (11.0)	0.080 (62.5)
8640		0.045 (13.0)	0.080 (62.5)
10080*		0.038 (11.0)	0.081 (62.9)

Table 3.4 Specific Activities of Immobilised Trypsin Samples Incubated for Various Times at 60°C. Percentage activity relative to zero time shown in brackets.
1 day
* 7 days

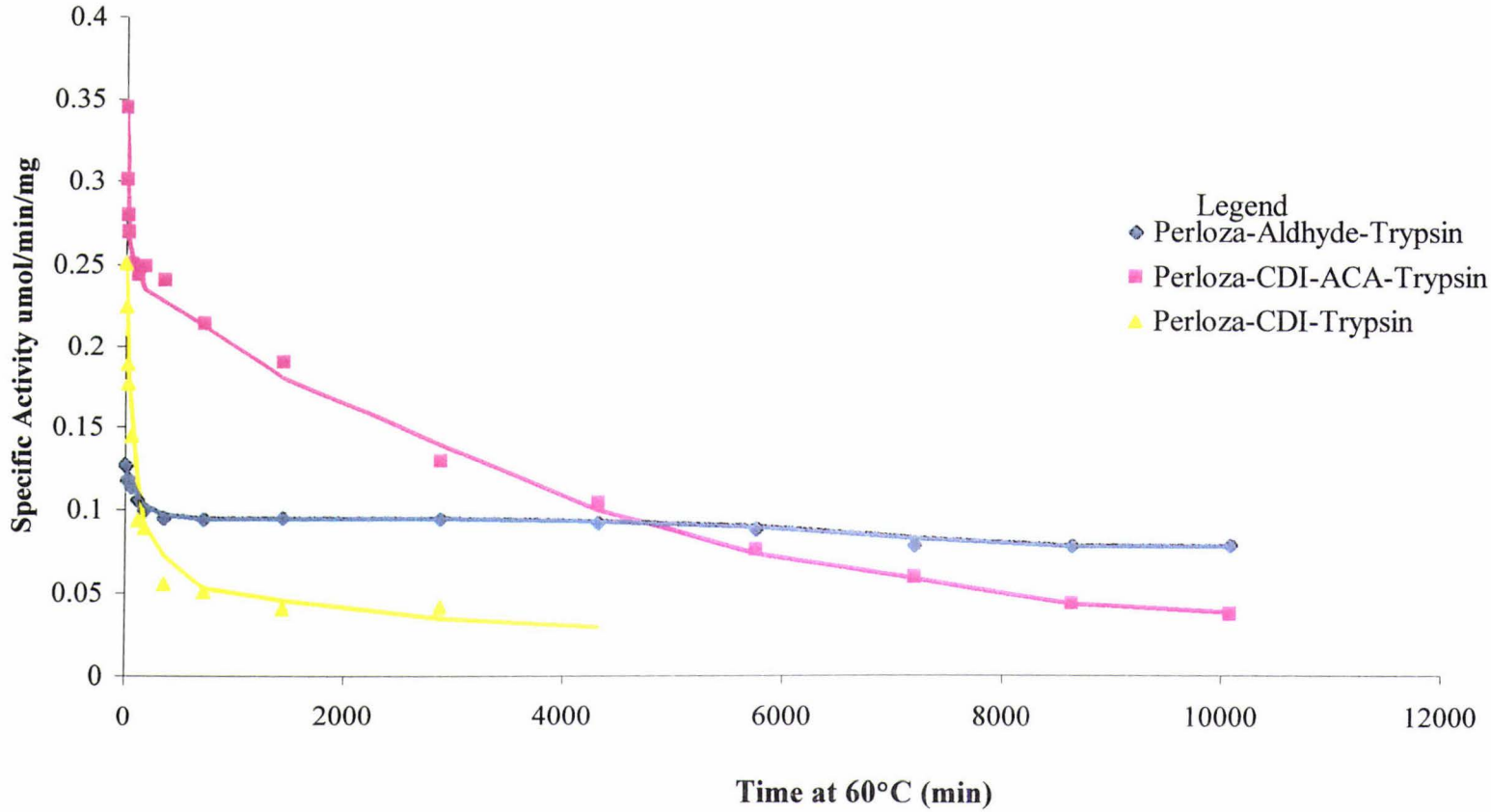


Figure 3.3 Specific Activities of Immobilised Trypsin Samples versus Time Incubated at 60°C.

Trypsinolysis of Synthetic Peptide

Each of the immobilised trypsin samples plus free trypsin was studied using a synthetic peptide as substrate (Figure 3.1b). Controls of each trypsin sample alone, peptide alone and the reaction mixture alone were also run. The samples were then injected onto an HPLC column and run from 100% buffer A, 0% buffer B to 40% buffer A, 60% buffer B. Buffer A consisted of 98% water, 2% acetonitrile, 0.1% TFA, buffer B consisted of 98% acetonitrile, 2% water, 0.1% TFA. The main peaks were then collected for amino acid analysis (AAA) and mass spectrometry.

Original peptide showed one main peak at 31% B. After overnight trypsinolysis HPLC analysis for each sample showed two main peaks plus several smaller peaks. The first main peak at 16% B contained peptide **b** and the second at 34% B contained peptide **a** (Figures 3.4 and 3.5). Initially it was thought the pattern of smaller peaks may be from trypsin autolysis. However control samples of each trypsin sample without peptide gave no peaks by HPLC, but a pattern of peaks similar to the minor ones on trypsinolysis samples was found in a control analysis of peptide only.

Amino acid analysis of the two major peaks gave similar results for each trypsin sample (Tables 3.5 and 3.6). Both agreed relatively well with expected values. In each of the samples aspartic acid in peak I was lower than expected, the reason for this was unknown. In two of the samples threonine was high possibly due to an inaccurate baseline. In peak II results glycine was low in two samples; again this was possibly due to an inaccurate baseline. In both cases of inaccurate baseline the amino acid analyser was not working well therefore subsequent samples were analysed on another machine. In two samples cysteine was not found and in another it was very low. However this was not surprising as cysteine is often partially or completely destroyed during acidic hydrolysis.

Analysis by mass spectrometry showed all peak I samples to have a mass of 733.3 Da (Figure 3.6). All peak II samples had a mass of 623.5 Da (Figure 3.7). These results for peak I mass correspond well with calculated results of 732.9 Da for peak I and 624.0 Da for peak II.

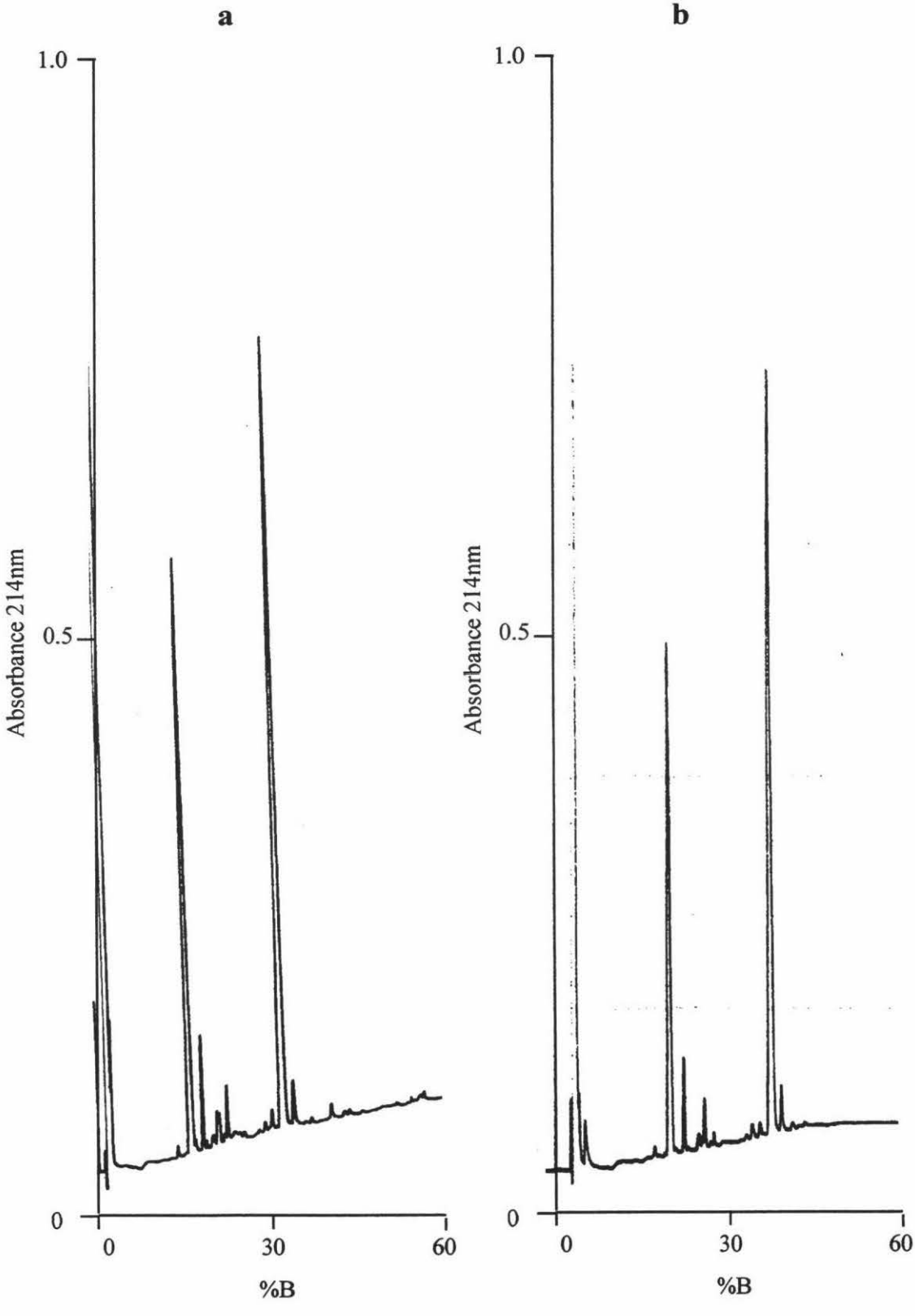


Figure 3.4 Trypsinolysis HPLC a) Free Trypsin b) CDI-Trypsin

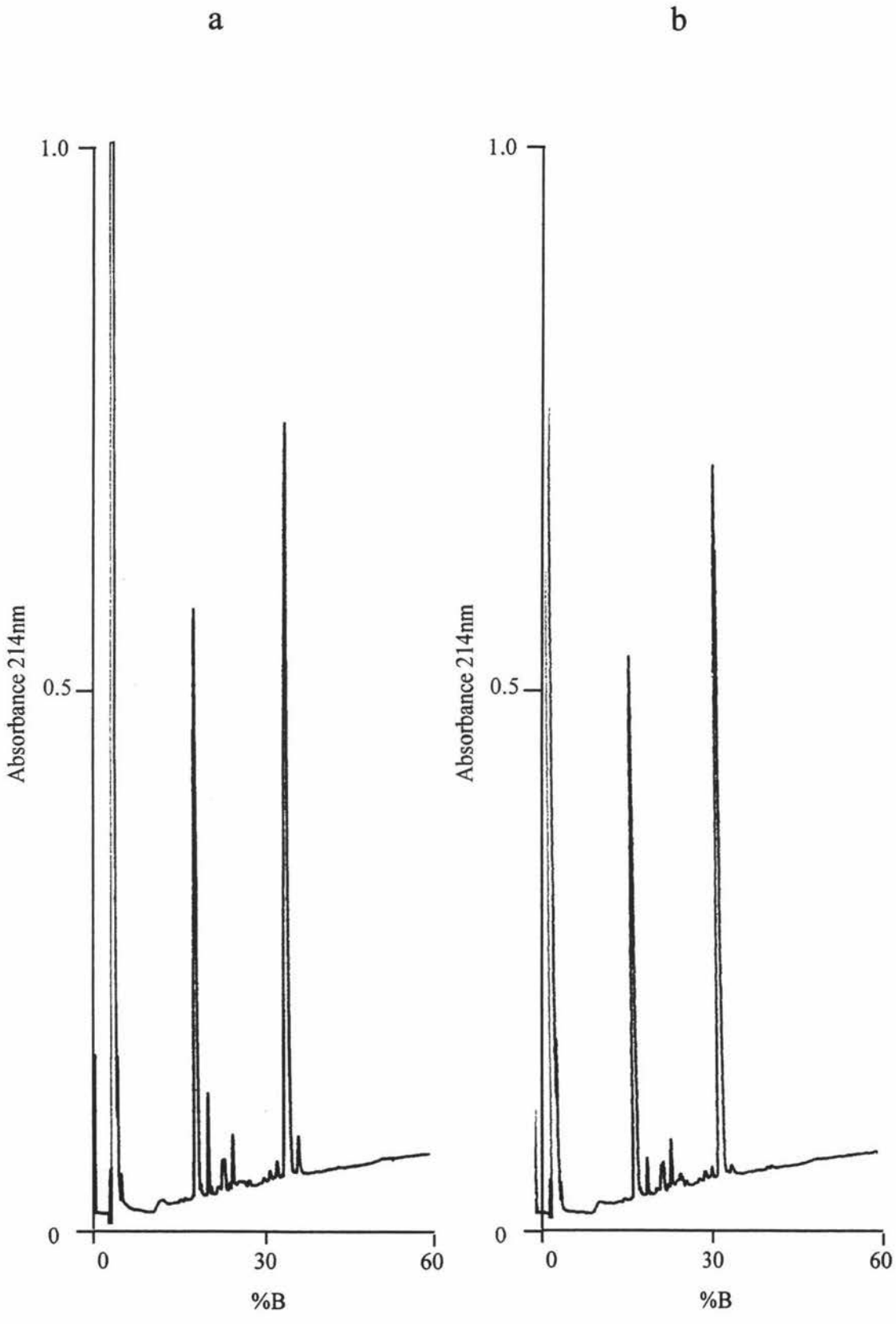


Figure 3.5 Trypsinolysis HPLC a) CDI-ACA-Trypsin b) Diol/Aldehyde-Trypsin

Amino Acid	Expected Number	Free Trypsin	CDI-Trypsin	CDI-ACA-Trypsin	Aldehyde-Trypsin
Aspartic Acid	2	1.38	2.29	1.45	1.92
Isoleucine	1	1.15	1.08	1.07	1.12
Threonine	1	0.96	0.97	1.32	1.26
Alanine*	2	2.00	2.00	2.00	2.00
Lysine	1	0.97	1.00	1.07	1.07

Table 3.5 Amino Acid Analysis Results of Peak I (peptide **a**)
*Analysis results worked out relative to Alanine

Amino Acid	Expected Number	Free Trypsin	CDI-Trypsin	CDI-ACA-Trypsin	Aldehyde-Trypsin
Phenylalanine	1	1.07	0.92	0.84	0.92
Isoleucine	1	0.92	0.96	0.78	0.95
Glycine	3	2.15	2.70	2.41	2.92
Alanine*	1	1.00	1.00	1.00	1.00
Cystine	1	0.31	-	-	-

Table 3.6 Amino Acid Analysis of Peak II (peptide **b**)
*Analysis worked out relative to Alanine

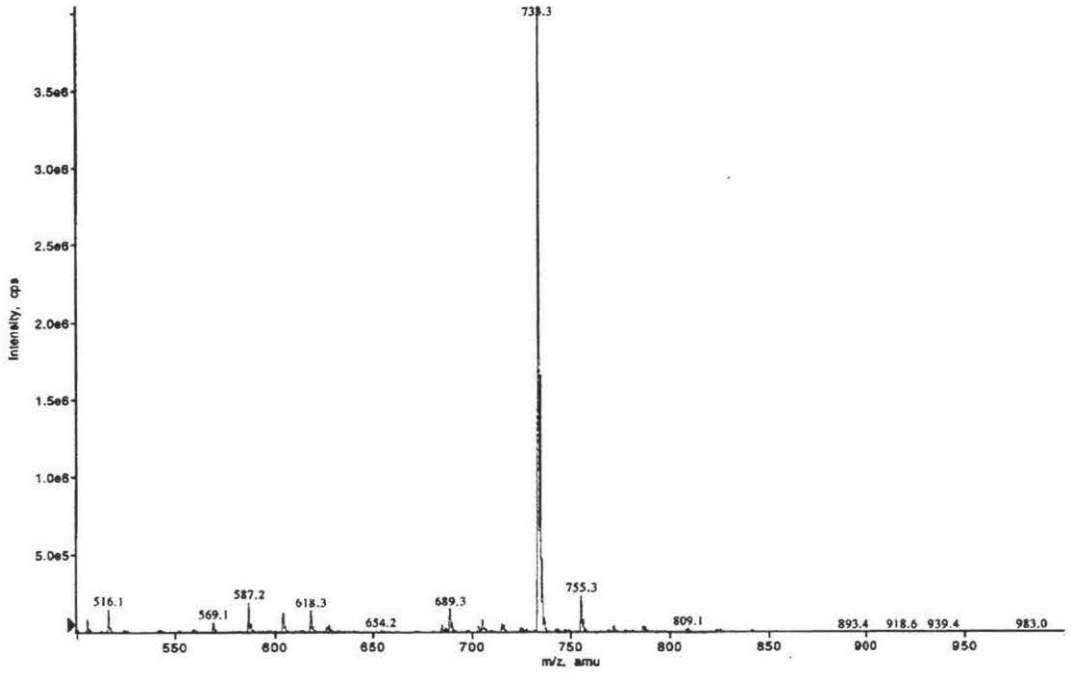


Figure 3.6 Mass Spectrometry for Perloza™-CDI-ACA-Trypsin Peak I (peptide **a**)

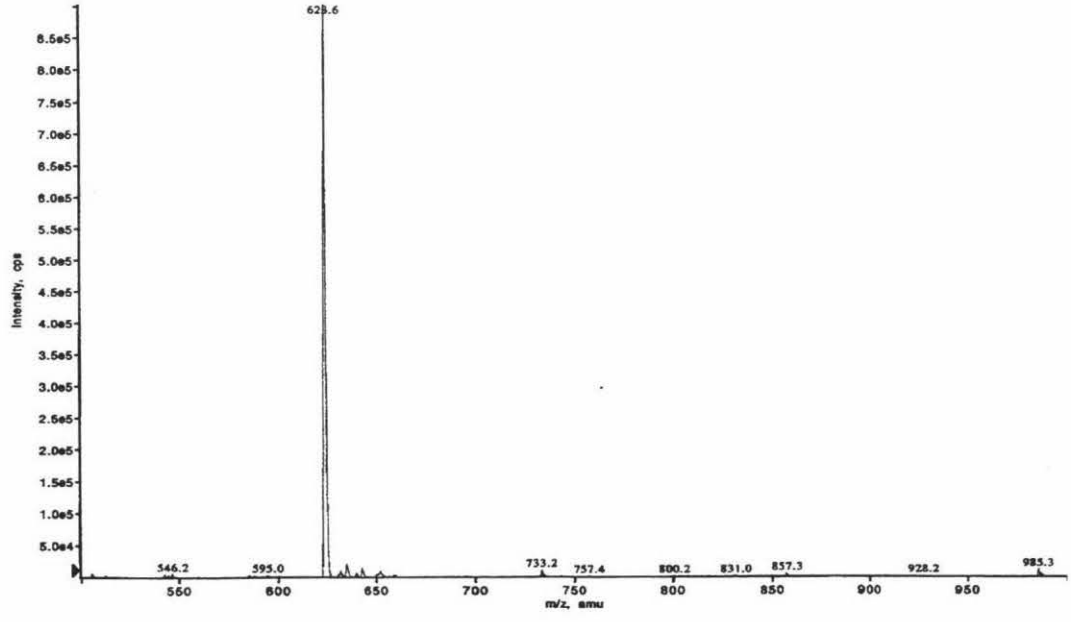


Figure 3.7 Mass Spectrometry for Perloza™-CDI-ACA-Trypsin Peak II (peptide **b**)

Conclusions

To obtain a comparison of immobilisation chemistries used on Perloza™, trypsin as a cheap, useful and readily available enzyme was selected for initial studies. Three immobilisation methods were utilised with significant success. All three methods, CDI, CDI/ACA and diol/aldehyde were employed with lower substitution levels than used previously for the immobilisation of smaller ligands. Substitution levels of the activating species were between 0.250 and 0.280mmol/g dry resin for Perloza™-CDI, 0.137mmol/g dry resin for Perloza™-CDI-ACA and between 0.270 and 0.295mmol/g dry resin for Perloza™-Aldehyde. These led to trypsin substitution levels of between 2.61 and 3.00mg/g wet resin for Perloza™-CDI, 2.94mg/g wet resin for Perloza™-CDI-ACA and 7.72mg/g wet for Perloza™-Aldehyde. These trypsin substitutions gave good initial specific activities.

A major problem with enzyme immobilisation by covalent binding is the loss of activity after immobilisation. Initial CDI-Trypsin activity was less than 2% below free trypsin initial activity. CDI-ACA-Trypsin had 71.5% of the activity of free trypsin and aldehyde-Trypsin had 36.5% of the activity of free trypsin.

Free trypsin lost almost 90% of its activity after only 10 minutes at 60°C. Perloza™-CDI-Trypsin showed a sharp drop in activity initially, then activity loss levelled out, to 90% activity lost after 2 days at 60°C. Perloza™-CDI-ACA-Trypsin immobilised on resins with a similar substitution to CDI resins gave erratic results but with the lower substituted resin results were more reproducible, possibly due to there being less interaction between trypsin molecules as they would be less densely packed on the resin. Perloza™-CDI-ACA-Trypsin showed a sharp initial loss of activity and then a gradual decrease until after 7 days more than 90% of initial activity still remained. Perloza™-Aldehyde-Trypsin also showed a sharp loss in activity but levelled out to lose less than 40% of its original activity after 7 days at 60°C. Enzyme substitution of Perloza™-Diol/Aldehyde was similar to Perloza™-CDI-ACA-Trypsin, but problems with erratic results were not encountered with Perloza™-Aldehyde-Trypsin.

Qualitatively Perloza™-CDI-Trypsin, Perloza™-CDI-ACA-Trypsin, Perloza™-Aldehyde-Trypsin and free trypsin appeared to have the same activity towards a more natural peptide substrate as results for mass spectrometry and amino acid analysis were very similar.

All three preparations showed trypsin-like activity towards a "real" substrate. Perloza™-CDI-ACA-Trypsin showed higher activity retained after immobilisation than the other two preparations but gave a slightly lower retained activity after 7 days at 60°C than did Perloza™-Diol/Aldehyde-Trypsin. Perloza™-CDI-Trypsin retained more activity than Perloza™-Diol/Aldehyde-Trypsin and less than Perloza™-CDI-ACA-Trypsin but lost activity more quickly than both. Perloza™-Diol/Aldehyde-Trypsin performed best in the 60°C heat trials as after an initial sharp drop retained activity changed very little over 7 days. In preparing Perloza™-CDI-Trypsin and Perloza™-CDI-ACA-Trypsin significant amounts of organic solvents were used which are potential health hazards, difficult to dispose of and expensive. Preparation of Perloza™-Diol/Aldehyde-Trypsin used aqueous chemistry making it a cleaner, safer less expensive alternative to the CDI/ACA chemistries for possible use in industry.

Trypsin is a widely used enzyme in several industries and in this study was used as a model for the immobilisation of other enzymes on Perloza™ using the available chemistries.

CHAPTER FOUR

CHYMOTRYPSIN

Introduction

Chymotrypsin belongs to the chymotrypsin/trypsin family of serine proteases. It is synthesised as an inactive zymogen in the acinous cells of the pancreas in mammals and is then carried to the small intestine in pancreatic juice for conversion to the active enzyme (Hess, 1971). Activation of chymotrypsin from its zymogen is by tryptic cleavage and subsequent autolysis. As with other members of the serine protease family it contains a reactive serine at its active site. Its natural substrates are proteins and peptides. Chymotrypsin cleaves adjacent to the carbonyl group of the aromatic amino acids phenylalanine, tyrosine and tryptophan and more slowly at the hydrophobic amino acids methionine, histidine and leucine (Blow, 1971).

The bovine α-chymotrypsin used in this study had a molecular weight of 25,000 and 241 amino acids (Hess, 1971), 14 of which were lysine residues, potential attachment sites for attachment with CDI, ACA and diol/aldeyde activation methods. Again resin substitutions were kept relatively low to reduce attachment at multiple sites on the enzyme.

Previous methods for chymotrypsin immobilisation include entrapment in a polyacrylamide gel (Kuan *et al*, 1980), covalent bonding (Sun *et al*, 1996) and adsorption (Låås, 1975).

Chymotrypsin A is most stable at pH 3.0 but autolysis still may occur slowly. At pHs above 10.0 and below 3.0 chymotrypsin undergoes a reversible conformational change. Above pH 5.0 extensive autolysis occurs (Wilcox, 1970).

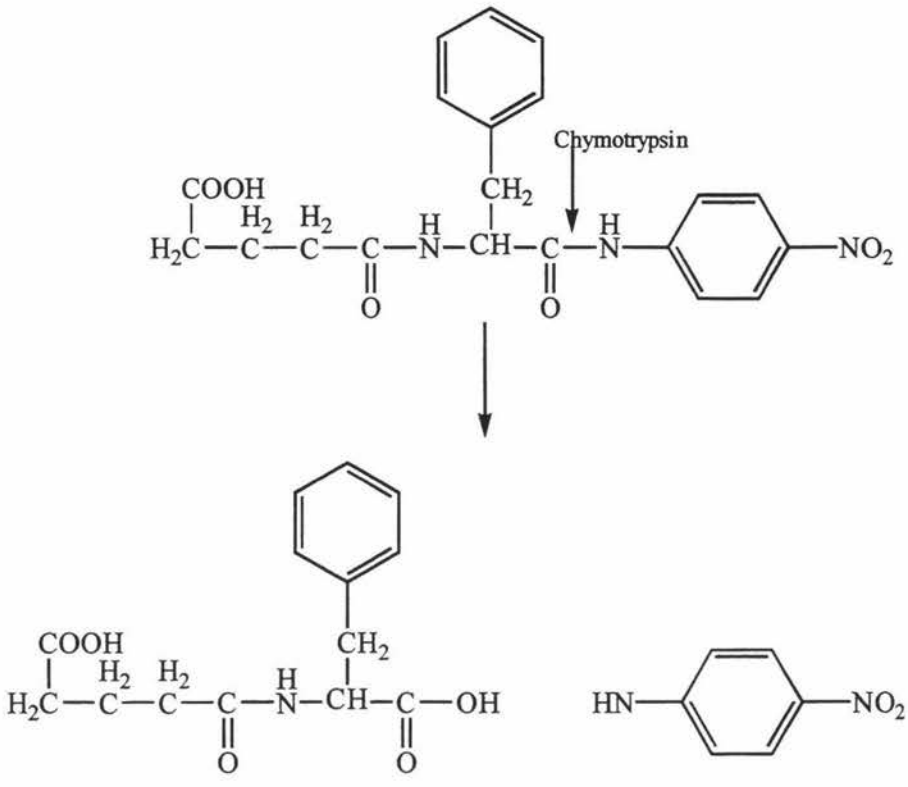


Figure 4.1a GAPNA Assay for Chymotrypsin Activity

Chymotrypsin cleaves the carboxyamide bond to produce *p*-nitroaniline which absorbs at 410nm.

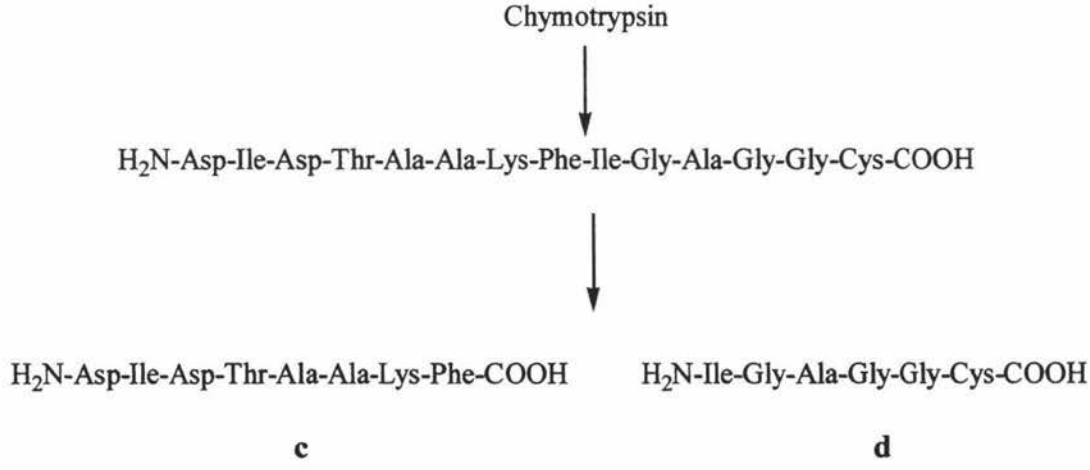


Figure 4.1b Chymotrypsinolysis of "Real" Substrate

Results and Discussion

Conditions for Perloza™ activation were the same as for trypsin. Enzyme attachment conditions were also similar to trypsin. Chymotrypsin activity was determined from the GAPNA assay, similar to the BAPNA assay for trypsin. Chymotrypsin acts on the artificial chromogenic substrate by cleaving the amide bond to release the ρ-nitroanaline chromophore (Figure 4.1a). Digests were also performed on the same synthetic peptide as used for trypsinolysis to give an example of "real" activity (Figure 4.1b). This time the peptide should be cleaved at phenylalanine to again result in two smaller peptides, an 8mer **c** and a 6mer **d** separable by HPLC and distinguished by AAA and mass spectrometry.

Activation of Perloza™

CDI activation of Perloza™ for both direct chymotrypsin immobilisation and via ACA was as for trypsin. CDI substitution levels were between 0.22 and 0.28 mmol/g dry resin. As for the trypsin study substitution levels were low i.e. designed to minimise the possibility of multiple attachment at several sites on the enzyme. ACA substitution levels were between 0.22 and 0.25 mmol/g dry resin also similar to trypsin. Aldehyde substitution was 0.295mmol/g dry resin.

Enzyme Attachments

Samples of 1g wet Perloza™-CDI were incubated for various times between 30 minutes and 2 hours to obtain the optimum time for chymotrypsin immobilisation. Most chymotrypsin appeared to be attached after 1 hour (Table 4.1). An incubation time of 1 hour was selected for attachments directly to CDI resins and via ACA spacer arm. Enzyme substitutions levels for larger scale attachments used in 60°C heat studies were between 1.08-1.49 mg/g wet resin for Perloza™-CDI and between 7.46-7.70 mg/g wet resin for Perloza™-CDI-ACA. Samples (1g wet resin) of aldehyde resins were incubated for 1 hour and 16 hours with chymotrypsin to find the optimum attachment time. Aldehyde resins used for immobilisation of smaller ligands were incubated for 16 hours (Burton, 1996). Chymotrypsin substitution after 1 hour

(7.70mg/g wet resin) was higher than the substitution after 16 hours (6.65mg/g wet resin) even though the same resin was used for attachment (substitution = 0.295 mmol/g dry resin), the same solution of enzyme was used and incubations were started at the same time. Possibly some autolysis may have lowered the amount of chymotrypsin present. Chymotrypsin substitutions for the large scale attachments were between 1.08 and 1.49 mg/g wet resin for Perloza™-CDI, 7.64 and 7.70 mg/g wet resin for Perloza™-CDI-ACA and between 5.76 and 6.55 mg/g wet resin for Perloza™-Aldehyde resins.

Incubation Time (min)	Substitution (mg/g wet)
30	1.41
60	1.80
90	1.92
120	2.50

Table 4.1 Chymotrypsin Substitution Levels on Perloza™-CDI with Variable Incubation Times. CDI Substitution = 0.258 mmol/g dry resin.

GAPNA Assay

Initial GAPNA assays were based on methods from Walters (1986) for chymotrypsin immobilised via CDI and CDI/ACA. The assay method was similar to trypsin; a suspension of resin sample was pipetted into the reaction mix. This gave inconsistent results so resin samples were subsequently weighed into vials. Later heat studies for chymotrypsin immobilised on CDI, CDI/ACA and aldehyde Perloza™ used the assay method in Huckle et al, 1996. The assay buffer was thus altered from 0.05M Tris.HCl, 0.05M $CaCl_2$ (pH 8.0) to that used by Huckel et al (1996) which was 0.05M Tris.HCl (pH 8.0). Control activity assays were also done with each activated resin without enzyme attached.

Heat Study at 60℃

Initial results using the assay method of Walter (1986) showed 15% retained activity after 3 hours for Perloza™-CDI-Chymotrypsin (Figure 4.2a) and 11% retained activity after 6 hours for Perloza™-CDI-ACA-Chymotrypsin (Figure 4.2b).

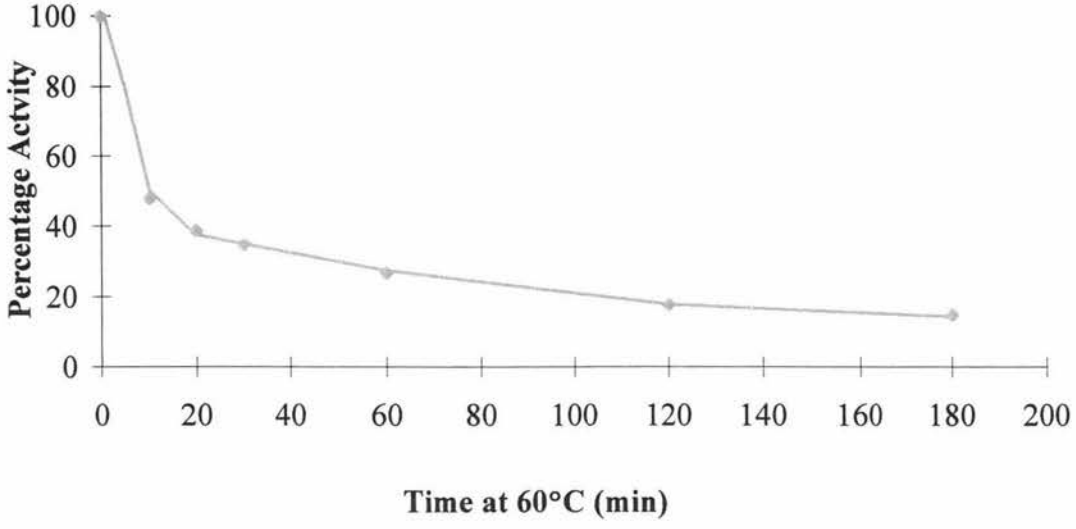
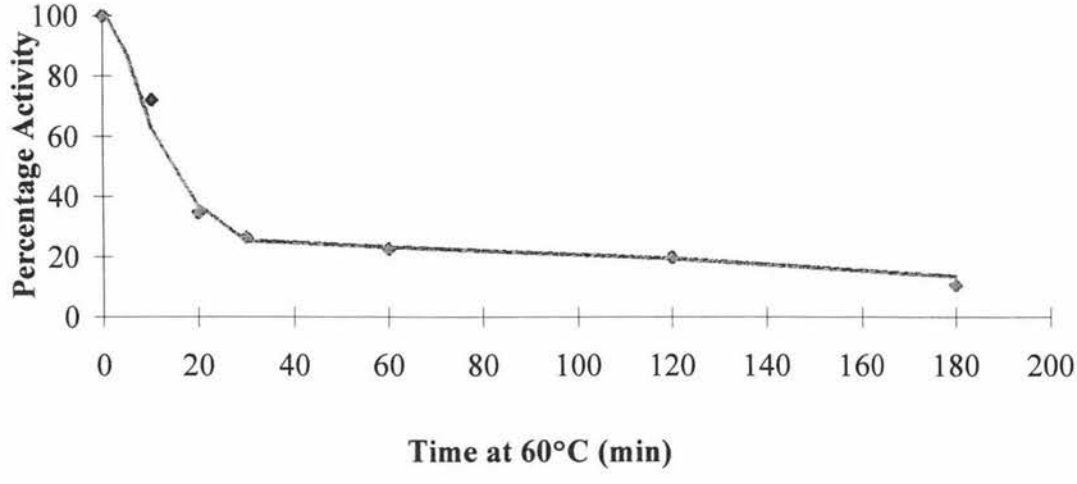


Figure 4.2a Composite Results From Preliminary Perloza™-CDI-Chymotrypsin Heat Study at 60°C



4.2b Composite Results from Preliminary Perloza™-CDI-ACA-Chymotrypsin Heat Study at 60°C

Chymotrypsin attached to Perloza™-CDI was incubated at 60°C for times ranging from 10 minutes to 3 hours (Figure 4.3, Table 4.2). Initial specific activity was 0.045 μmol min^{-1} mg^{-1}. This dropped sharply after only 10 minutes to 26.2% of original activity. Activity loss slowed and dropped to 17.2% of zero time activity after 3 hours. Chymotrypsin attached to Perloza™-CDI-ACA was incubated at 60°C for times ranging from 10 minutes to 6 hours (Figure 4.3, Table 4.2). Specific activity started lower than Perloza™-CDI-Chymotrypsin at 0.025μmol/min/mg and showed a

similar drop in activity to 27.0% after 10 minutes. Activity loss again slowed and dropped to 13.1% of zero time activity after 6 hours. Perloza™-Aldehyde-Chymotrypsin initial specific activity and loss of activity was similar to Perloza™-CDI-ACA-Chymotrypsin (Figure 4.3, Table 4.2). Specific activity started at 0.023 μmol min^{-1} mg^{-1} and dropped to 30.5% after 10 minutes and then to 14.7% after 6 hours. Free chymotrypsin lost most of its activity after 10 minutes at 60°C.

Time at 60°C (min)	Perloza™-CDI-Chymotrypsin (μmol min^{-1} mg^{-1})	Perloza™-CDI-ACA-Chymotrypsin (μmol min^{-1} mg^{-1})	Perloza™-Aldehyde-Chymotrypsin (μmol min^{-1} mg^{-1})
0	0.0449 (100)	0.0248 (100)	0.0228 (100)
10	0.0118 (26.3)	0.0067 (27.0)	0.0070 (30.7)
20	0.0115 (25.6)	0.0054 (21.8)	0.0061 (26.8)
30	0.0104 (23.2)	0.0044 (17.7)	0.0055 (24.1)
60	0.0098 (21.8)	0.0039 (15.7)	0.0046 (20.2)
120	0.0091 (20.3)	0.0036 (14.5)	0.0045 (19.7)
180	0.0077 (17.1)	0.0032 (12.9)	0.0040 (17.5)
360*		0.0033 (13.3)	0.0034 (14.9)

Table 4.2 Specific Activities of Immobilised Chymotrypsin Samples Incubated for various times at 60°C.
Percentage activity relative to zero time shown in brackets.
* 6hours.

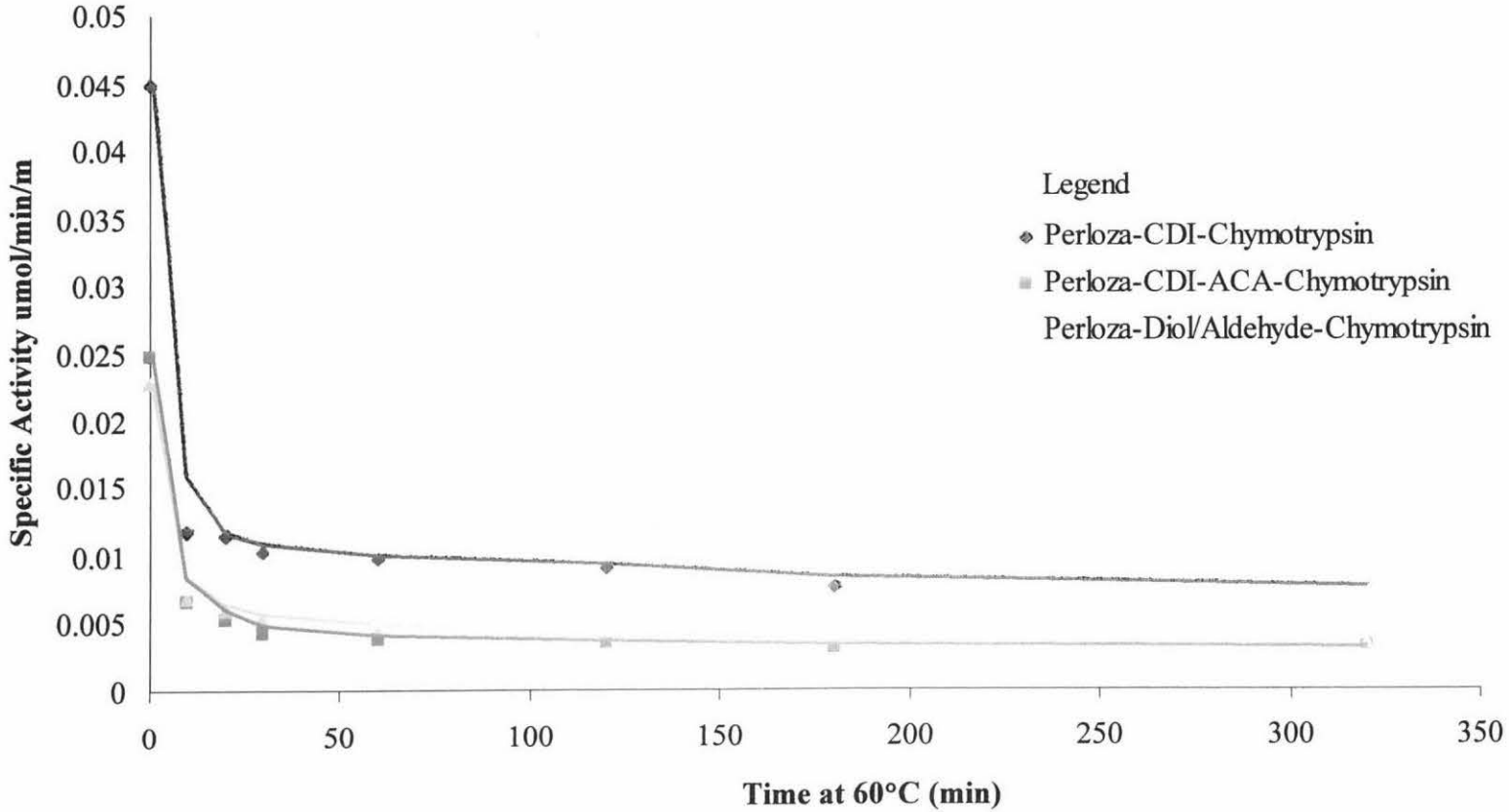


Figure 4.3 Specific Activities of Immobilised Chymotrypsin Samples versus Time Incubated at 60°C.

Chymotrypsinolysis of Synthetic Peptide

Immobilised chymotrypsin samples and free chymotrypsin were studied using the same synthetic peptide substrate and conditions as in the trypsinolysis study. Controls as in the trypsinolysis study were also run. After overnight reaction samples were injected onto an HPLC column, conditions were the same as for the trypsinolysis samples. The main peaks were collected for AAA and mass spectrometry.

After overnight reaction, HPLC analysis showed two main peaks. Peak I at 26% HPLC buffer B contained peptide **d** and peak II at 32% HPLC buffer B contained peptide **c** (Figures 4.4 and 4.5). AAA of the two peaks showed all results corresponded well with both the free chymotrypsin results and expected results (Tables 4.3 and 4.4).

Analysis by mass spectrometry showed all peak I samples to have a mass of 476.4 Da (Figure 4.6) and all peak II samples to have a mass of 880.3 Da (Figure 4.7). Peak II samples also showed a large peak at 440.9 Da. These results corresponded well with calculated values of 476.8 Da for peak I and 880.1 Da for peak II. The mass spectrometry trace shown is from Perloza™-CDI-ACA-Chymotrypsin. Traces from other samples were very similar.

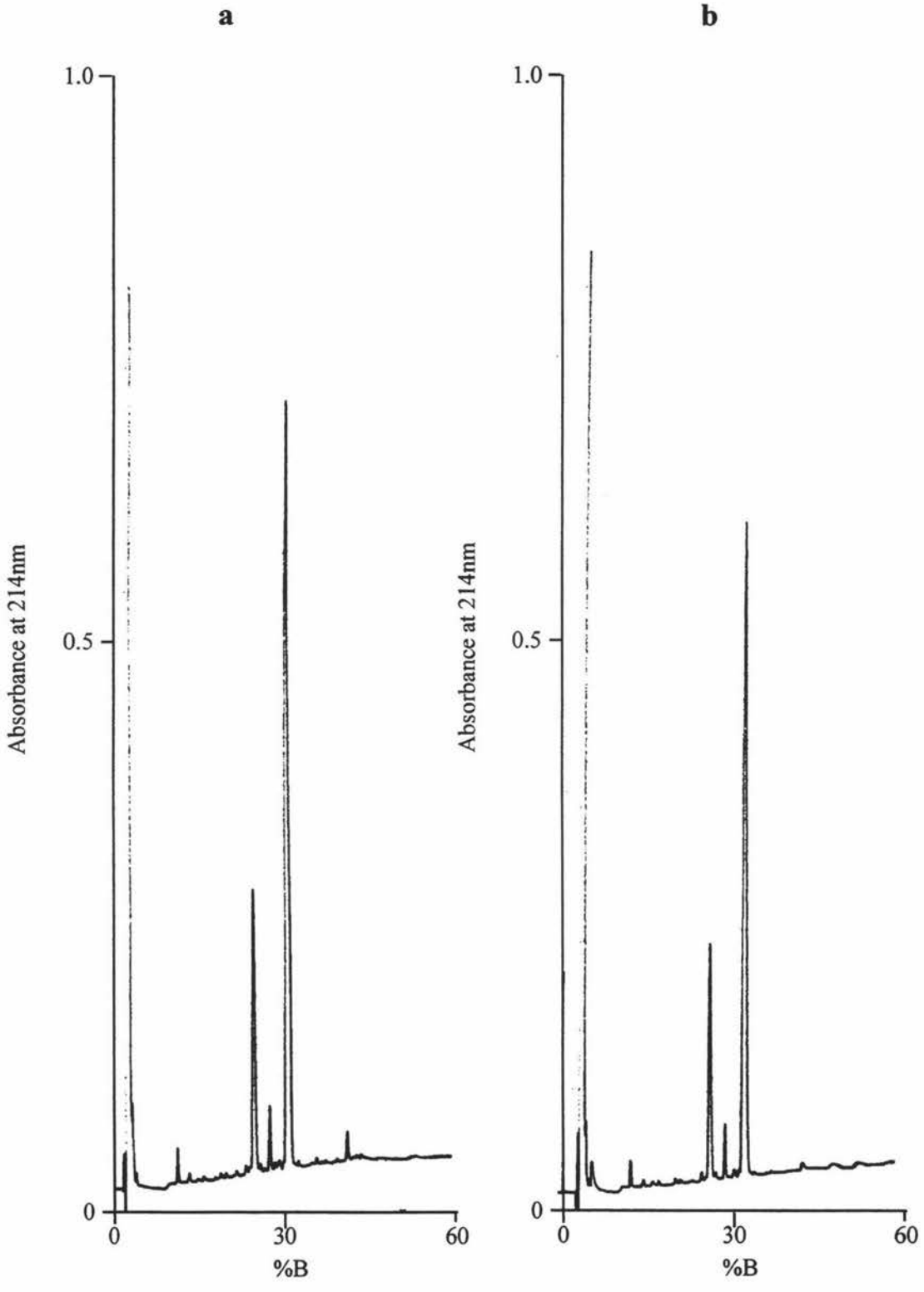


Figure 4.4 Chymotrypsinolysis HPLC Traces

a) Free Chymotrypsin **b)** CDI-Chymotrypsin

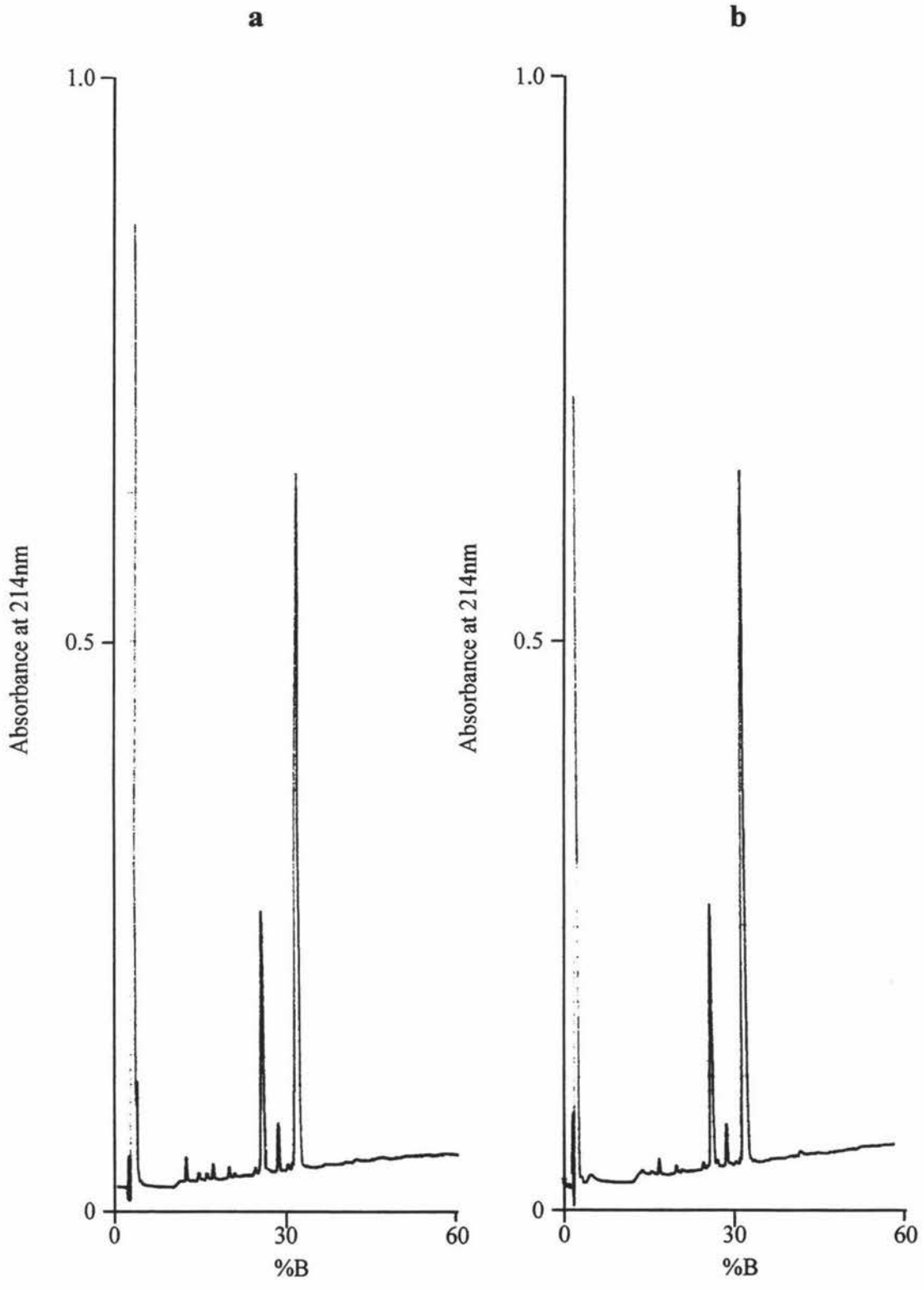


Figure 4.5 Chymotrypsinolysis HPLC Traces

a) CDI-ACA-Chymotrypsin **b)** Diol/Aldehyde-Chymotrypsin

Amino Acid	Expected Number	Free Chymotrypsin	CDI-Chymotrypsin	CDI-ACA-Chymotrypsin	Aldehyde-Chymotrypsin
Isoleucine	1	1.04	1.02	1.01	0.99
Glycine	3	2.79	2.80	2.70	2.79
Alanine*	1	1.00	1.00	1.00	1.00
Cysteine	1	-	-	-	-

Table 4.3 Amino Acid Analysis of Peak I (peptide **d**) (mol wt = 476.8)
*Analysis worked out relative to Alanine

Amino Acid	Expected Number	Free Chymotrypsin	CDI-Chymotrypsin	CDI-ACA-Chymotrypsin	Aldehyde-Chymotrypsin
Aspartic Acid	2	2.19	2.15	2.19	2.14
Isoleucine	1	1.05	1.11	1.11	1.09
Threonine	1	0.90	0.90	0.90	0.91
Alanine*	2	2.00	2.00	2.00	2.00
Phenylalanine	1	0.92	1.05	1.06	0.95
Lysine	1	0.98	1.07	1.01	1.01

Table 4.4 Amino Acid Analysis of Peak II (peptide **c**) (mol wt = 809)
*Analysis worked out relative to Alanine.

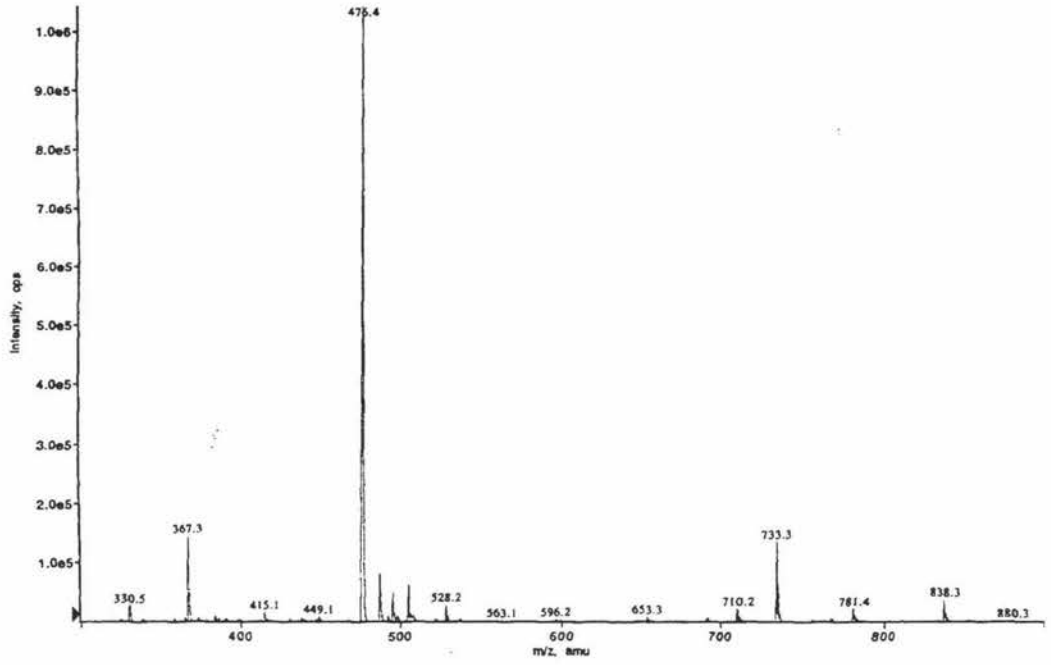


Figure 4.6 Mass Spectrometry for Perloza™-CDI-ACA-Chymotrypsin Peak I (peptide **d**)

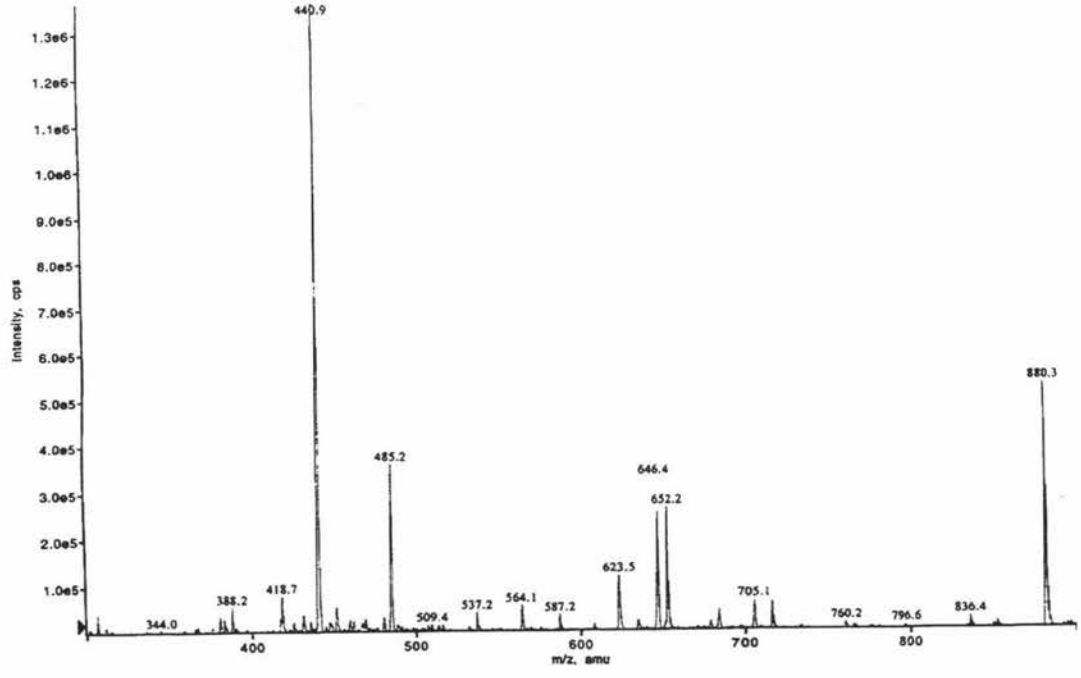


Figure 4.7 Mass Spectrometry for Perloza™-CDI-ACA-Chymotrypsin Peak II (peptide **c**)

Conclusions

Chymotrypsin, another proteolytic enzyme similar to trypsin, was also used in studies to obtain a comparison of the immobilisation chemistries used on Perloza™. Three immobilisation chemistries were used with some success. Each method utilised lower substitution levels than used to immobilise smaller ligands. Substitution levels of the activating species were between 0.22 and 0.25mmol/g dry resin for Perloza™-CDI, between 0.22 and 0.25mmol/g dry resin for Perloza™-CDI-ACA and 0.295mmol/g dry resin for Perloza™-Aldehyde. This gave protein substitution levels of between 1.08 and 1.49 mg/g wet resin for CDI resins, between 7.46 and 7.70 mg/g wet resin for CDI/ACA resins and between 5.76 and 6.55 mg/g wet resin for aldehyde resins. For CDI activated resins most of the enzyme seemed to be attached by 60 minutes and it was not thought necessary to allow the reaction to go any longer. Chymotrypsin immobilised on aldehyde resins gave a higher substitution after 1 hours incubation than 16 hours, therefore it was decided to incubate the larger scale samples for 1 hour.

After immobilisation Perloza™-CDI-Chymotrypsin had the highest initial specific activity followed by Perloza™-CDI-ACA-Chymotrypsin and then Perloza™-Aldehyde-Chymotrypsin. Heat studies at 60°C gave similar results in terms of percentage loss of activity with all three dropping to between 26.3 and 30.7% activity after 10 minutes, then levelling off to be between 12.9 and 17.5% after 3 hours. Immobilised chymotrypsin did not retain as much activity in the heat studies as immobilised trypsin, but some activity was still retained after incubation at 60 °C for 6 hours by Perloza™-CDI-Chymotrypsin and Perloza™-Diol/Aldehyde-Chymotrypsin. Perloza™-CDI-Chymotrypsin still retained activity after 3 hours at 60 °C. Free chymotrypsin lost all activity after 10 minutes at 60°C.

All three samples performed similarly in the heat studies. Perloza™-CDI-Chymotrypsin although having a higher specific activity than the other two samples had a much lower total activity due to its lower substitution which made it difficult to

measure accurately after 3 hours. Quantitative chymotrypsinolysis studies showed all three immobilised samples to have the same activity towards a "real" substrate by HPLC and mass spectral analysis. Results on HPLC also matched free chymotrypsin results and mass spectra weights corresponded well with expected values.

As for trypsin the Perloza™-Diol/Aldehyde-Chymotrypsin would have an advantage over the other two preparations in any industrial use due to the aqueous chemistry used in its synthesis.

CHAPTER FIVE

AMYLASE, HORSERADISH PEROXIDASE AND ALCOHOL DEHYDROGENASE

Introduction

α-Amylase

α-Amylase hydrolyses the α-1,4 linkages of amylose which is a major component of starch to give a mixture of maltose and glucose (Chang *et al,* 1993; Lehninger, 1977). It is found in micro-organisms, plants and mammals (Nakajima *et al,* 1986). α-Amylase is widely used in industry for brewing, bread making, paper making and in the textile industry (Yamane, 1989; Debabov, 1982).

α-Amylase is stable between pH 4.8 and 8.5 but is most active between pH 5 and 7 (Burton, 1995). The α-amylase used in this study was a recombinant variety from *Bacillus liceriformis* (courtesy of Genencor International, Palo Alto, California, USA) and had a molecular weight of 48 kDa.

α-Amylase activity is measured using the chromogenic substrate ρ-nitrophyenyl maltoheptaoside (BPNPG7) from Megazyme. α-Amylase cleaves the BPNPG7 to give a blocked maltosaccharide and ρ-nitrophenyl maltosaccharide. The ρ-nitrophenyl maltosaccharide is subsequently cleaved by glucoamylase and α-glucosidase to give ρ-nitrophenol which gives a yellow colour with a Trizma base that can be followed at 410nm (Figure 4.1a).

Horseradish Peroxidase

Peroxidases are found widely in the plant and animal kingdoms. Horseradish peroxidase (HRPO) is one of the most intensively studied peroxidases. It is found in the root of the horseradish plant. HRPO catalyses the conversion of peroxide to water. It is stable between pH 5 and 10, with a pH optimum of between 6.0-6.5. HRPO is a glycoprotein with a molecular weight of 44 KDa and contains a haem

group (Keesey, 1987). There are 6 lysine residues present (Welinder *et al,* 1972) as possible attachment sites using the three coupling chemistries. Immobilised preparations include attachment to attachment to agarose (D'Angiuro *et al*, 1987).

The activity assay used in this study converted pyrogallol to purpurogallin which can be followed at 420nm (Figure 4.1b)

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Alcohol Dehydrogenase

Alcohol Dehydrogenase (ADH) catalyses the conversion of ethanol and other alcohols to aldehydes. In mammals ADH is also involved in the metabolism of retinal. Yeast ADH shows more substrate specificity than mammalian ADH, where it is mainly involved with alcohol fermentation in the glycolytic pathway. The ADH used in this study is from Baker's yeast and has a molecular weight of 150kDa (Brändén *et al*, 1975). It is stable over a wide range of pHs but is most active between pH 8.5 and 9.5. Immobilised preparations include attachment of horse liver ADH to silica (Nilsson and Larsson, 1983) and attachment of yeast ADH to dextran (Fisher, 1997).

ADH activity was determined by following the production of NADH from NAD^+ at 340nm (Figure 4.1c). The assay used in this study is based on the method described by Cornell and Veech (1983).

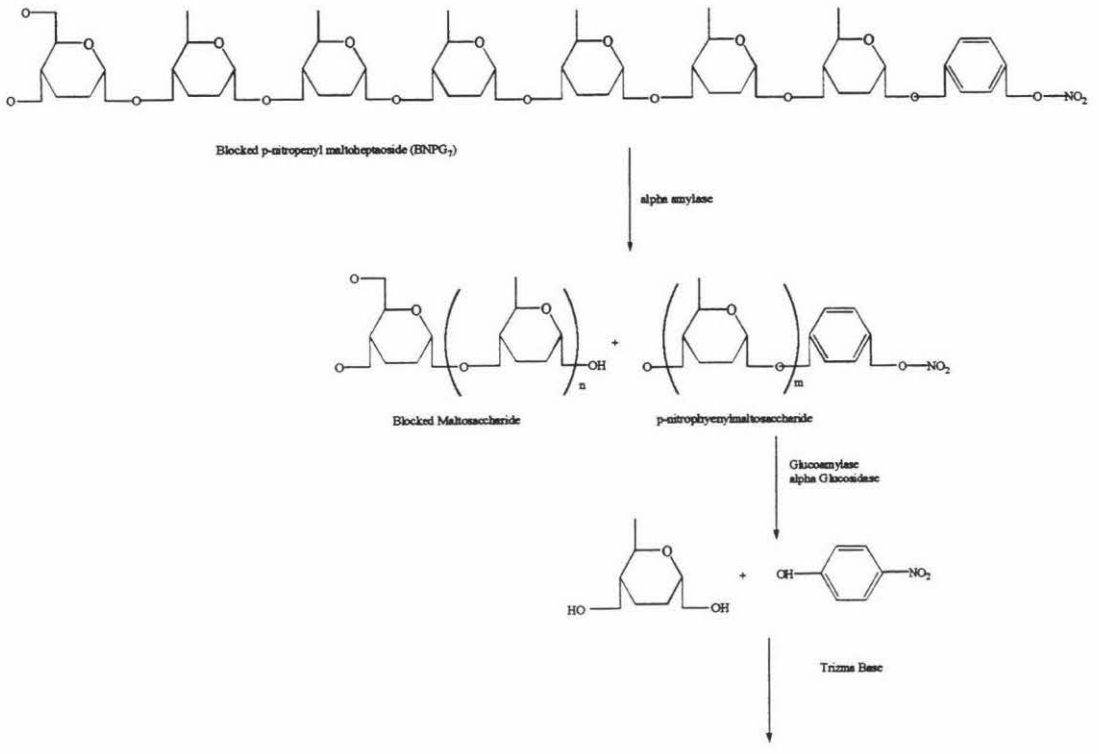

Figure 5.1a Activity Assay for α-Amylase (Megazyme)

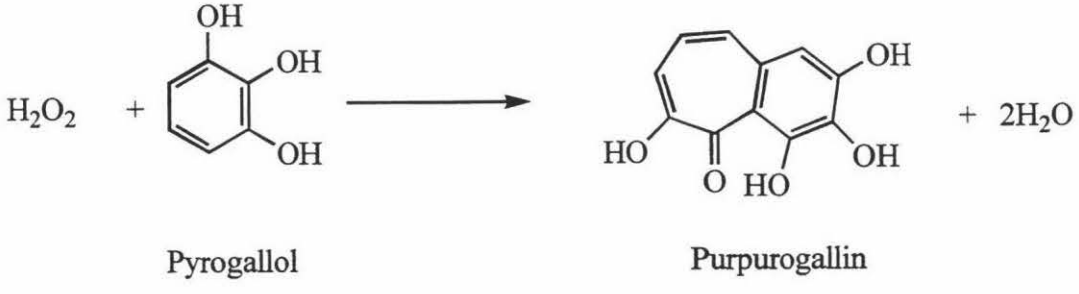

Figure 5.1b Activity Assay for Horseradish Peroxidase

Figure 5.1c Activity Assay for Alcohol Dehydrogenase

Results and Discussion

Activation of Perloza™

Activation using the three chemistries was as for the trypsin and chymotrypsin studies. Substitution levels were similar to previous studies, and are shown in Table 5.1

Activation Chemistry	α-Amylase	Horse Radish Peroxidase	Alcohol Deydrogenase
CDI	0.258	0.313	0.313
ACA	0.212	0.275	0.275
Diol/Aldehyde	0.296	0.311	0.311

Table 5.1 Substitution Levels for each Chemistry from Titration (results are shown in mmol/g dry resin)

Enzyme Attachments

α-Amylase attachments to Perloza™-CDI and Perloza™-CDI-ACA were carried out in 50 mM borate pH 9.0 (Burton, 1996). Attachments of α-amylase, ADH and HRPO to Perloza™-Diol/Aldehyde were similar to trypsin/chymotrypsin attachments. ADH and HRPO attachments to Perloza™-CDI and Perloza™-CDI-ACA were similar to trypsin chymotrypsin attachments. Substitution levels from BCA assays are shown in Table 5.2.

Activation Method	α-Amylase	Horse Radish Peroxidase	Alcohol Dehydrogenase
Perloza™-CDI	0.44-0.63	0.56-0.59	0.33-0.41
Perloza™-CDI-ACA	4.46-5.01	3.05-3.21	5.46-5.34
Perloza™-Diol/Aldehyde	3.68-4.20	4.62 and 5.01	3.85 and 4.41

Table 5.2 Enzyme Substitution Levels (BCA Assay). Results expressed as mg/g wet resin.

Activity Assays

All three enzymes had assays which ran for only 1-2 minutes. With free enzyme this involved the addition of all components to a cuvette, mixing and following the change of absorbance in the spectrophotometer. With immobilised enzymes to Perloza™ this is difficult as resin settles during the assay and interferes with readings. Initially with activity assays of these three enzymes, resin samples were filtered from the reaction mixture and the filtrate measured. Results were difficult to reproduce using this procedure as it was hard to get the timing precise. Therefore the assays were done as for non-immobilised samples with the resin-enzyme sample present in the cuvette. Specific activities are shown in Table 5.3. The compromise was to leave the resin in the cuvette for the assay but to run the assay for 1 minute, as with longer than 1 minute the settling of the resin became more of a problem. Control activity assays of each activated resin without enzyme attached were also run.

Resin	α-Amylase	Horse Radish Peroxidase	Alcohol Dehydrogenase
Perloza™-CDI	-	-	-
Perloza™-CDI-ACA	20.81	263.80	-
Perloza™-Diol/Aldhyde	7.19	390.07	-

Table 5.3 Specific Activities (expressed as μmol min^{-1} mg^{-1})

The Perloza™-CDI-ACA preparation for α-amylase gave the highest initial specific activity, which was nearly 3 times higher than the Perloza™-Diol/Aldeyde preparation. The activities of both preparations were much lower than the imitial activity for free α-amylase which was 358.7 μmol min^{-1} mg^{-1}. Preparations of immobilised HRPO followed a different pattern with the specific activity for the Perloza™-Diol/Aldeyde preparation being slightly higher than the specific activity for the Perloza™-CDI-ACA preparation. Specific activity for free HRPO was 2910.12 μmol min^{-1} mg^{-1}. For both α-amylase and HRPO there was no measurable activity for the Perloza™-CDI preparations. None of the ADH preparations gave measurable activity.

Conclusions

HRPO, α-amylase, and ADH were all successfully immobilised to Perloza™-CDI-ACA and Perloza™-Diol/Aldeyde. HRPO and α-amylase retained activity after immobilisation to each resin preparation. Compared with the free enzyme α-amylase immobilised on Perloza™-CDI-ACA retained 5.8% activity, while the α-amylase-Perloza™-Diol/Aldeyde preparation retained 2.0%. HRPO immobilised on Perloza™-

CDI-ACA retained 9.1% activity and 13.4% when immobilised on Perloza™-Diol/Aldeyde, compared with the free enzyme. Therefore immobilisation of these two larger enzymes was not as successful as the smaller enzymes, with trypsin and chymotrypsin retaining more activity after immobilisation. Attachment of HRPO, ADH and α-amylase to Perloza™-CDI gave very little success. Only very small amounts of proteins were immobilised and the preparations showed no activity by the assays used. The activated group may not have extended far enough from the resin or a longer coupling time than 1 hour may have been required. Perloza™-Diol/Aldeyde preparations have only one extra link in the spacer between enzyme and resin but gave much higher substitution levels. These Diol/Aldeyde preparations were incubated with the enzymes for longer than the CDI preparations, which may have explained the increased coupling yields relative to Perloza™-CDI.

All three enzymes were much larger than trypsin and chymotrypsin with ADH, a tetramer, being the largest at 150kDa. The fact that ADH was a tetramer may have led to there being no activity on immobilisation as the strong covalent bonding may have disrupted the weaker bonds holding the monomers together. This appeared to be the case in a previous study immobilising yeast ADH to dextran (Fisher, 1997). However horse liver ADH (a dimer) has been successfully immobilised previously using a diol activated silica (Nilsson and Larsson, 1983). HRPO and α-amylase showed retained activity indicating it was possible to immobilise larger enzymes to Perloza™ by the chemistries used in this study.

CHAPTER SIX

CONCLUSIONS AND FUTURE WORK

Conclusions

In the studies reported in Chapters 2-5 the immobilisation of enzymes to Perloza™ beaded cellulose was investigated using three different attachment chemistries. Enzyme immobilisation has been primarily considered for its use by industry and in medicine to solve problems caused by the lability of many enzymes, their lack of prolonged stability and the difficulty in many cases of separating enzyme from product (Melrose, 1971). Most immobilisation methods aid in the removal of protein from product (Rosevear, 1987) and some aid in stabilising the enzyme against adverse conditions (Klibanov, 1978 and 1983).

Perloza™ beaded cellulose resin was chosen as it has previously been derivatised with significant success for use in peptide synthesis (Englebretsen, 1992; Englebretsen and Harding 1992a and b; 1993; 1994a, b & c), and protein chromatography (Burton, 1995; Burton and Harding, 1997a and b; Burton *et al*, 1997). In the latter case the relatively cheap cost of Perloza™ and its stability at high flow rates allowed development of an application in large scale protein purification, in a fluidised bed chromatography system on the multikilogram scale (Genencor International, 1996). Several approaches for activation are available through the many hydroxyl groups of Perloza™.

Three methods of covalent binding were compared for the immobilisation of five enzymes in this study. The enzymes studied were trypsin, chymotrypsin, α-amylase, horseradish peroxidase and alcohol dehydrogenase with molecular weights of 23, 25, 48, 44 and 150 kDa respectively. They were investigated for their ability to bind to Perloza™, retention of catalytic activity and in the cases of trypsin and chymotrypsin their stability to heating to 60°C. All were easily immobilised using Perloza™-CDI-ACA and Perloza™-Diol/Aldehyde and all except alcohol dehydrogenase gave significant levels of retained activity. Immobilisation to Perloza™-CDI gave much

lower levels of enzyme substitution especially for α-amylase, horse radish peroxidase and alcohol dehydrogenase, but nevertheless trypsin and chymotrypsin gave measurable activities after immobilisation to Perloza™-CDI.

Immobilisation by any method can be seen to produce a number of scenarios varying from zero through single to multiple attachment. In other words the enzyme could be

A. trapped or temporarily caught and accessible to smaller molecules such as BAPNA but **not** covalently bound to the matrix. The result would be a sharp drop in activity as the enzyme gained no stability from immobilisation and is either diluted out of the matrix and/or autolysed (Figure 6.1).
B. covalently bound, accessible but with relatively few bonds between enzyme and support giving some stability due to increased rigidity compare with the free enzyme leading to a medium rate of inactivation upon heating (Figure 6.1).
C. covalently bound, accessible with a relatively higher number of bonds between enzyme and support, giving greater rigidity to the enzyme compared with the free enzyme and so a lower initial activity but also and a slower drop in activity (Figure 6.1) (Klibanov, 1979).

Therefor initial enzyme activity is not necessarily the primary consideration when contemplating applications, in particular industrial applications of immobilised enzymes. Reliable long-term activity of the immobilised enzyme is more desirable than high initial activity followed by a sharp drop or a continuous gradual decline in activity. In addition, even if the activity of the immobilised enzyme is much lower than the activity of the free enzyme, for industrial use lower levels of activity may be acceptable provided that long-term activity is maintained. Multiple surface lysines and the N-terminus with varying pKa's may be available for attachment so a variety of orientations for any particular enzyme will be possible. It should therefore not be unreasonable to expect different immobilisation methods to give different proportions of these populations.

Using the above 3 scenarios we can then speculate that the sharp initial drop shown by Perloza™-CDI-Trypsin (Figure 3.2), and the three chymotrypsin preparations

(CDI, CDI/ACA and Diol/Aldehyde, Figure 4.3) indicated a high initial population of trapped non-covalently bonded enzyme, A and/or some B. Differences in initial activity between the chymotrypsin samples could be explained by variations in accessibility to the active site evolving from A, B and/or C. The subsequent levelling out of activity indicated another population of enzyme covalently bound by several bonds, C. Perloza™-Diol/Aldehyde-Trypsin (Figure 3.3) showed a smaller percentage drop than the other preparations and then a levelling which indicated the possibility of a higher initial proportion of enzyme covalently bound by several bonds (C). The enzyme substitution of Perloza™-CDI-ACA-Trypsin was similar to Perloza™-Diol/Aldehyde-Trypsin but gave a graph indicating a combination of all three possible scenarios i.e. a heterogeneous population of enzyme (Figure 1.2).

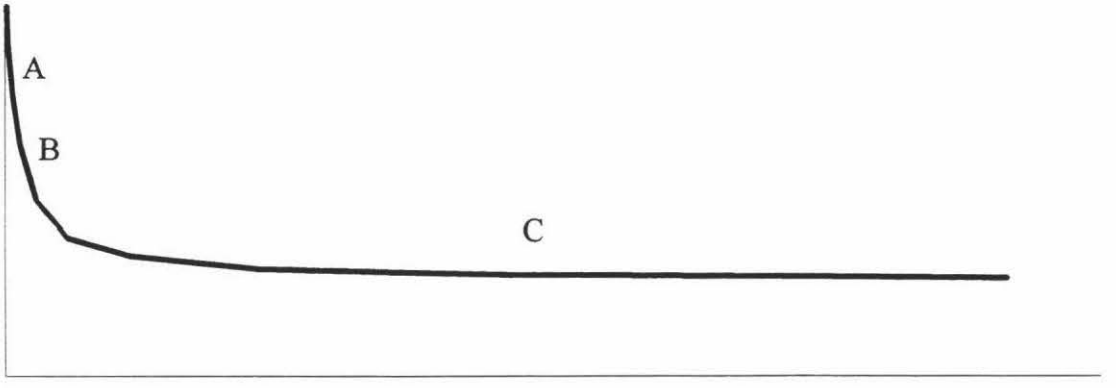

Figure 6.1 Immobilisation Scenarios

Horseradish peroxidase, alcohol dehydrogenase and α-amylase are all larger enzymes than trypsin and chymotrypsin, therefor the incidence of trapped uncovalently bound enzyme in Perloza™ pores should be reduced, especially for ADH. This led to the lower substitution levels found for the Perloza™-CDI preparations of these three enzymes.

From this limited study, it can be seen that smaller enzymes such as trypsin and chymotrypsin, can be successfully immobilised to Perloza™ with retention of their normal activity and increased stability especially in the case of the Perloza™-Diol/Aldehyde-Trypsin. Larger enzymes such as HRPO and α-amylase have also

been shown to be successfully immobilised with retention of activity, but immobilisation can disrupt the bonds between subunits of enzymes such as alcohol dehydrogenase.

Future Work

Investigations on trypsin and chymotrypsin were more extensive in this study than the other three enzymes. Therefore studies on heat stability for HRPO could be done using the Perloza™-CDI-ACA and Perloza™-Diol/Aldehyde preparation to investigate the possibility of stabilisation of larger enzymes. The α-amylase used in this study is already quite heat stable (Burton, 1996). As horse liver ADH has already been immobilised successfully on silica (Nilsson and Larsson, 1983) it could be used in further studies as an example for immobilisation of multisubunit enzymes to Perloza™.

For each enzyme optimisation of the substitution level could be done. Substitution of activating groups at a lower level than in this study and at a higher level would allow for a compromise between the lower initial activity and increased stability. A lower level of substitution may suit a multisubunit enzyme such as ADH as there may be less disruption of bonds between subunits. A previous study immobilising yeast ADH encountered similar problems with activity (Fisher, 1997).

Other chemistries developed for soft gel carbohydrate matrices (such as agarose/Separose™) may be useful in improving the performance of chymotrypsin and ADH on Perloza™. Preparations such as an allyl glycidyl ether epoxide substituted Perloza™ resin may be useful, as they would lead to attachment of enzymes through carboxyl containing amino acids such as aspartic acid, glutamic acid and the C-terminal carboxyl group rather than lysine and N-terminal amine groups may be useful. Immobilisation using an epoxide substituted resin with either an amine or thiol linked spacer arm containing a carboxyl group could also be used. A water soluble carbodiimide can then be used in either of the preceding cases to couple the target enzyme.

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